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THE

MICROTOMIST'S VADE-MECUM

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A HANDBOOK OF THE METHODS OF MICROSCOPIC ANATOMY

BY

ARTHUR BOLLES LEE

LONDON
J. & A. CHURCHILL
11, NEW BURLINGTON STREET
1885

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PREFACE

In its primary intention this work appeals rather to the instructed anatomist than to the beginner. Its aim is to put into his hands a concise but complete account of all the methods of preparation that have been recommended as useful for the purposes of Microscopic Anatomy, and so furnish him with a ready source of information on points of detail as to which his memory or his knowledge may be at fault. This object is attained by the mere collection of Formulæ set out in Part I, and of special methods described in Part II. But the book could obviously be made to subserve a further end—that of a guide to the beginner. To this end I have added a General Introduction and a series of introductory paragraphs prefixed, where needful, to the different chapters. These introductory portions, taken together, go far to make up a formal treatise on the art. And as a further aid to the beginner I have added the collection of examples given in Part II. These examples are of course not intended for servile imitation, but rather as hints suggestive of the most fitting processes.

The collection of Formulæ here brought together is, I believe, practically exhaustive; no process having any claim to scientific status having been rejected, nor any, I trust, unwittingly omitted. It may be useful here to say a word as to the reasons for this—perhaps apparently excessive—catholicity of treatment. Doubtless a large proportion of the formulæ given are quite superseded in modern practice; but that is not a sufficient reason for rejecting them. The inclusion of all of them is justified by the consideration that some one or other of them may perhaps serve, in some way that cannot now be foreseen, to suggest some new method of value. Let me give an example. Who, ten years ago, would have thought that the formula of Blanchard's 'Liqueur saline hydrargyrique' deserved reprinting in a treatise on histologic technic? Yet it is to the disinterment of that forgotten formula by Lang that we owe the establishment of corrosive sublimate as one of the most useful fixing agents in the arsenal of the microtomist. Or who would have deemed Thiersch's lilac borax-carmine (Formula No. 80*a*), published in 1865, to be of greater importance than any other stain till then made known? Yet that formula it was that directly suggested Woodward's admirable aqueous borax-carmine, and through this, if I am not mistaken, the aqueous and the alcoholic borax-carmines of Grenacher, the latter of which is now to be found on the table of every embryologist.

All my abstracts and translations have been made from the original sources, except where it has been impossible for me to obtain sight of these. References to the sources are given in all cases; but I desire here to make special acknowledgement of the great assistance rendered me by the Journal of the Royal Microscopical Society—in many respects the best-edited periodical known to me.

GENEVA (SWITZERLAND);
February, 1885.

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PART I

THE MICROTOMIST'S VADE-MECUM.

1

CHAPTER I.

INTRODUCTORY.

1. From a superficial inspection of the formulæ set out in the following pages, an uninformed person might not unreasonably conclude that microscopical methods are as numerous as microscopical anatomists, or even that to every particular investigation there belongs a particular process; he would not suspect that there is a backbone of consentaneous practice running through modern researches. That conclusion, however, would be erroneous; such a backbone exists; the great majority of recent investigations have been mainly carried out by carefully *fixing* the structures to be examined, *staining* them with a *nuclear* stain, *dehydrating* with alcohol, and mounting *series of sections* of the structures in balsam. Or, to put the matter in another way, modern zootomists are generally agreed as to the desirability of working with carefully-fixed tissues (a thing which never entered the heads of the old school), of working with dehydrated objects instead of with objects soaked in any of the aqueous preservative media of the old school, and of working with series of sections made with a microtome, instead of working with objects teased or squeezed or otherwise "dissociated," as histologists of the old school were obliged to do in pre-microtome times. And the agreement as to this practice is not absent from matters of detail; the great majority of preparations are made by fixing either with sublimate or a picric acid combination, washing-out with alcohol, staining with alcoholic borax-carmine, imbedding in chloroform-paraffin, cutting with a sliding microtome, and mounting the sections in series in Canada balsam. By this, which may be called the general or normal method, the work is blocked-out and very often finished; special points being studied, if necessary, by special methods, such as examination of the living tissue elements *in sitû*, or in "indifferent" media; fixation with more precise fixing agents, such as osmic acid or some chromic mixture; staining with more precise nuclear stains; dissociation by teasing or maceration. Practices antithetical to these principles, such as throwing living tissues into weak glycerine and leaving them to die and macerate there, or making multiple stains with gaudy but diffusely-staining anilins, are not scientific, and are only employed by dilettanti or by persons very ignorant of histology. The freezing microtome is almost exclusively used by dilettanti and pathologists; I have never seen one employed by a zoologist, nor met with any account of a zootomical discovery made by means of it. Such exceptions as might be quoted do not invalidate the position that the method I have called general or normal really is such; and as such I now proceed to describe it in sufficient detail to enable any reader who is unacquainted with it to study it for himself. The special methods will be found sufficiently described under their respective headings, so that it does not appear useful to write a general introduction to them here.

2. The first thing to be done with any structure is to *fix* its histological elements. Two things are implied by the word "fixing;" first, the rapid killing of the element, so that it may not have time to change the form it had during life, but is fixed in death in the attitude it normally had during life; and second, the hardening of it to such a degree as may enable it to resist without further change of form the action of the reagents with which it may subsequently be treated. Too much stress can hardly be laid on this point, which is the most distinctive feature of modern histological practice; without good fixation it is impossible to get good stains, or good sections, or preparations good in any way.

The most convenient fixing agents are picro-sulphuric acid and corrosive sublimate. The structure having been duly treated with one of these, or with some other (for details concerning the employment of these reagents see the chapter on FIXING AGENTS), is to be washed in order to remove from the tissues as far as possible all traces of the fixing reagent. If corrosive sublimate, or osmic acid, or a solution into which chromic acid or a chromate enters, has been used for fixing, the washing may be done with water. But if picric acid in any form has been used, the washing must be done with alcohol. The reason of this difference is that the first-named reagents (and indeed all the compounds of the heavy metals used for fixing) appear to enter into a state of chemical combination with the elements of tissues, rendering them insoluble in water; so that the hardening induced by these agents is not removed by subsequent treatment with water. Picric acid, on the other hand, produces only a very slight hardening of the tissues, and does not appear to enter into any combination whatever with their elements, as it is entirely removable by treating the tissues with water or alcohol. If the removal be effected by means of water, the tissue elements are left in a soft state in which they are obnoxious to all the hurtful effects of water. Alcohol must therefore be taken to remove the picric acid, and to effect the necessary hardening at the same time.

At the same time that the superfluous fixing agent is being removed from the tissues, or as soon as that is done, the water of the tissues must be removed. This is necessary for two reasons; firstly in the interest of preservation, the presence of water being the condition of all others that most favours post-mortem decomposition; and secondly, because all water must be removed in order to allow the tissues to be impregnated with the paraffin necessary for section-cutting, or with the balsam in which they are to be finally preserved. (The cases in which aqueous imbedding and preserving media are employed are exceptional, and will be treated of in the proper places.) This *dehydration* is performed as follows: the objects are brought *gradually* into alcohol, which is effected by transferring them into it by means of a small spoon or a pipette, so that they carry with them a little atmosphere of aqueous fluid into which the surrounding alcohol gradually diffuses. They are then passed through successive alcohols of gradually increased strength, for instance, 50 per cent. two hours, 70 per cent. six to twenty-four hours, 80 per cent. several hours, 95 per cent. two or three hours, absolute alcohol, time enough for complete saturation. (Very small objects, so small that section-cutting is not necessary, may be dehydrated much quicker than this. Infusoria may be prepared in a few minutes.) The water having been thus completely removed, the object is brought into good chloroform, where it remains till saturated, which generally happens in a few minutes in the case of fairly small objects. The chloroform is now to be gradually saturated with paraffin. This is done by placing it, with the object, on a water-bath, heating it to the melting point of the paraffin employed, and dropping into it from time to time small pieces of paraffin. When it is seen that no more bubbles are given off from the object the addition of paraffin may cease, as that is a sign that the paraffin solution has entirely taken the place of the chloroform in the object. This displacement having been gradual, the risk of shrinkage of the tissues is reduced to a minimum. The heating is then continued (at the melting point of the pure paraffin) until the whole of the chloroform has been driven off, which may be conveniently tested by the smell. The object is then imbedded in a convenient position, and sections are cut (*see* IMBEDDING METHODS). The sections, cut dry, are mounted in series on a slide by the method of Frenzel, or by that of Schällibaum, or by that of Mayer (*see* SERIAL SECTION METHODS). The paraffin is now removed and the sections are stained, generally with borax-carmine, which two operations are performed as follows: A series of glass tubes large enough to hold a slide is filled with the following reagents, and arranged in the following order: Turpentine (or naphtha); absolute alcohol; 90 per cent. alcohol; 70 per cent. alcohol; alcoholic borax-carmine; 70 per cent. alcohol acidulated with HCl; 90 per cent. alcohol; absolute alcohol. The slide, having been warmed to the melting point of the paraffin, is plunged into the turpentine, which removes the paraffin; then passed through the tubes with the successive alcohols into the stain, from which it is brought into the successive alcohols of the ascending series, which wash out the stain and dehydrate the sections. Nothing more now remains to be done but to treat the sections with a drop of benzol or turpentine, and to add Canada balsam and a covering glass.

The plan of staining sections on the slide is of very recent introduction; before it had been worked out the practice was to stain structures *in toto*, before cutting sections. And in cases in which structures are sufficiently small and permeable to allow of satisfactory staining in this way, and if it be not essential to save time, this plan is quite as good as the one described. In this case the object after having been fixed and washed out is taken while still on its way through the lower alcohols (it should not be allowed to proceed to the higher grades of alcohol before staining) and passed through a bath of alcoholic borax-carmine (or other alcoholic stain) of sufficient duration, then dehydrated with successive alcohols, passed through chloroform into paraffin, and cut as above described,[1] the sections in this case being mounted direct from the turpentine or naphtha with which the paraffin is removed. If aqueous staining media be employed (and it is sometimes very desirable for particular purposes to prepare specimens with alum-carmine or picro-carmine) the structures should either be stained *in toto* immediately after fixing and washing out, or sections may be stained on the slide in the manner above described, a bath of 35 per cent. alcohol being intercalated between the 70 per cent. alcohol and the aqueous stain, and between the latter and the ascending series of alcohols.

The treatment of objects which can be studied without being cut into sections is identical with that above described, with the omission of those passages that relate to the treatment with paraffin. Its normal course may be described as fixation with sublimate or picro-sulphuric acid, washing out with alcohol, staining with alcoholic borax-carmine, treatment with successive alcohols of the grades above named, final dehydration with absolute alcohol, clearing with clove oil, and mounting in balsam. The remarks above made as to the employment of aqueous stains apply to this case, these stains being employed, as a rule, before the treatment with alcohol; it being a maxim that an object that has once been in alcohol should not be allowed to go back into water, if that can possibly be avoided.

In the case of structures that are intact and covered by an integument not easily permeable by liquids special care must be taken to avoid shrinkage from exosmosis on the passage from the last alcohol into oil of cloves. A slit should be made in the integument, if possible, so that the two fluids may mingle without hindrance. And in all cases the passage is made gradual by placing the oil of cloves (or other clearing medium) *under* the alcohol. This is done as follows: A sufficient quantity of alcohol is placed in a tube (a watch-glass will do, but tubes are generally better), and then with a pipette a sufficient quantity of clove oil is introduced *at the bottom of the alcohol.* The two fluids mingle but slowly. The objects to be cleared being now quietly put into the supernatant alcohol, float at the surface of separation of the two fluids, the exchange of fluids takes place gradually, and the objects slowly sink down into the lower layer. When they have sunk to the bottom, the alcohol may be drawn off with a pipette, and the objects examined in the clearing medium, or mounted in balsam. (It may be noted here that this method of making the passage from one fluid to another applies to all cases in which objects have to be transferred from a lighter to a denser fluid, for instance, from alcohol, or from water, to glycerine.)

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[1] If the objects have already been soaked in clove oil, or other essential oil, for the purpose of clearing, they may either be imbedded direct from the clove oil, or this may be removed by means of chloroform, which is the better practice.

3. The Naples Methods.[1]—It may be useful here to give an account of the methods actually employed in the Zoological Station of Naples, both in order to bear out the statements I have made regarding the general method of research, and in order to call attention to one or two points which it is necessary to take into consideration in the study of marine organisms.

Fixation is always carefully attended to. Pure alcohol is not very suitable as a fixing agent, as it precipitates most of the salts of the sea-water adhering to the surface of marine animals, thus giving rise to a crust that prevents the penetration of the alcohol to the interior (thus allowing maceration of the internal structures to be set up), and also hinders the penetration of staining fluids. Besides this, it often gives rise to a coagulation of the perivisceral fluids that glues the viscera together in a most undesirable manner. Therefore, if alcohol is used, *acidulated* alcohol is generally taken (*see* FIXING AGENTS). But the fluid most used is Kleinenberg's picro-sulphuric acid (*see* FIXING AGENTS). The great advantages of this fluid are that it kills quickly, that it removes the sea-salts from the tissues, and that it allows of good staining subsequently. For special cases picro-nitric or picro-hydrochloric acid is used instead (*see* FIXING AGENTS). Osmic acid is used, but only for very permeable tissues (on account of its very feeble penetrating power), and in cases in which it is not important that a subsequent nuclear stain should be easily attainable (*see* FIXING AGENTS). Chromic acid is also avoided as much as possible on account of its hindrance to staining (*see* FIXING AGENTS). Combined with platinum chloride however, in the mixture known as Merkel's fluid, it is said to allow of good staining, and is found useful in some special cases (*see* FIXING AGENTS). Corrosive sublimate is found very convenient, and is the fluid most used, excepting picro-sulphuric acid (*see* FIXING AGENTS).

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[1] Mayer, in 'Mitth. Zool. Stat. Neapel,' ii (1881), p. 1, *et seq.* See also the abstract in 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), pp. 866—881, and that in 'Amer. Natural,' xvi (1882), pp. 697—706, in which two last some improvements are mentioned which have been worked out since the publication of Mayer's paper.

Washing out is done with successive alcohols, water being used only in the case of corrosive sublimate, osmic acid, or the chromic mixtures. Staining is done in almost all cases with alcoholic staining media, and these are made to contain if possible about 70 to 80 per cent. alcohol (not more, because it is found that fluids containing much more than that proportion of alcohol stain more diffusely than solutions weaker in alcohol). The stains most used are Grenacher's borax-carmine, Mayer's modification of Grenacher's alcoholic carmine, Kleinenberg's hæmatoxylin, and Mayer's cochineal (for all these *see* STAINING AGENTS). Anilin stains are only used in special cases, viz. as nuclear stains by the Hermann-Böttcher process (*see* STAINING AGENTS, ANILIN STAINS).

Aqueous stains are more seldom used, but alum-carmine and picro-carmine are frequently found very useful. Eosin is only used for double staining in combination with picro-carmine (*see* STAINING AGENTS, **Picro-carmine and Eosin**). All stains containing ammonia are most carefully avoided.

Imbedding is generally done with chloroform-paraffin; wax-and-oil, "after Brücke's plan," being sometimes preferred for very delicate objects. Calberla and Selenka's albumen imbedding process is abandoned, partly on account of the aqueous nature of the process, and partly on account of the long immersion in the *warm* liquid that is necessary. Sections are generally stained on the slide after having been fixed in position by one of the methods above mentioned; but many objects are still stained *in toto* before cutting.

Clearing is done with clove oil, cannel oil, bergamot oil, or turpentine according to circumstances (*see* CLEARING AGENTS). Minute dissections are done, if necessary, in a drop of the clearing agent (*see* CLEARING AGENTS, **Clove Oil**). Mounting is done in chloroform-balsam, or in colophonium (*see* EXAMINATION MEDIA). Aqueous mounting media are very seldom employed, for the two reasons, that to get thoroughly rid of the water of an object is the thing most essential to the permanent preservation of tissues, and that " it is seldom that it is not possible to mount an object in balsam so as to show more than can be seen in any glycerin or other aqueous preparation."

It is important that the reasons for employing alcoholic rather than aqueous staining media be rightly understood. Since, in most cases, treatment with alcohol forms part of the fixing process, alcoholic solutions are logically indicated for staining. For by means of them it is possible to avoid the bad effects that follow on passing delicate tissues from alcohol into water, violent diffusive currents being thereby set up which sometimes carry away whole groups of cells; swellings being caused in the elements of the tissues; and, if the immersion in the aqueous medium be prolonged, as is generally necessary in order to obtain a thorough stain, maceration of the tissues supervening. But alcoholic staining fluids have still other advantages; they are vastly more penetrating; with them alone is it possible to stain through chitinous integuments; they stain more quickly; the stain as a rule is more precise; and, if it be desired to stain slowly, tissues may be left in them for days without hurt.

There are a few exceptional cases in which aqueous staining media are admissible, and some in which they are preferable to alcoholic media. Thus Mayer finds that picro-carmine is less hurtful to tissues than other aqueous stains, and attributes the result to the picrin contained in the solution; and other workers have found that alum-carmine gives very good results when employed after alcohol. And in general with tissues that have been well hardened in chromic acid the action of aqueous fluids does not appear to be seriously hurtful. Mayer observes that with certain groups of animals it is necessary to allow the tissues to be reimbibed and swelled up with a watery fluid in order to obtain the required action of the colouring agent. Lang finds that Turbellaria can only be successfully stained by means of an aqueous solution of picro-carmine and eosin.

CHAPTER II.

FIXING AGENTS.

4. By the fixing of tissues is meant the rapid killing of their elements, together with such a degree of hardening as suffices to fix them in their natural forms, and enable them without distortion or other injury to undergo such further treatment with staining agents or other chemical reagents, such hardening or clarifying processes, as may be desirable. I do not, as some writers appear to do, restrict the meaning of the term to the instantaneous killing and hardening of protoplasm alone, but include also under it a preliminary hardening of the formed tissue-elements, fibres, tubes, membranes, and so forth. An example or two will make evident both the meaning of the term and the importance of the process in all researches into delicate structures. In the course of their beautiful inquiry into the "Development and Retrogression of the Fat-cell" (*vide* 'Journ. Roy. Mic. Soc.,' vol. xi, p. 353) the Hoggans investigated the relations subsisting between the wandering cells found on serous membranes and the fat-cells also found there. They chose as an object of study the growing broad ligament of pregnant rats and mice. They drenched the animals with chloroform, with the idea of anæsthetising the individual cells of its tissues, they stretched the portion of membrane to be examined over vulcanite rings, and treated it with solution of nitrate of silver, in order not only to fix "the various cells forming the membrane in the condition or shape they possessed during life," but also in

order that "the shoals of wandering cells, which are continually groping over the free surfaces of the abdominal organs," might be firmly fixed *in sitû* on the membrane. They were thus (they claim) able to show that fat-cells are specially developed from wandering cells, "which may appear to be round or branched cells, according to the process by which they have been prepared." The authors state that at first they injected the animal, but gave up that practice on finding that the loss of time caused them to miss "some most valuable indications." It must, I think, be evident that in such a case as this, if the observer does not take care to kill the cells of his tissue instantaneously, but allows them to die at leisure (as would happen, for instance, if he began by injecting a carmine-gelatin solution and waited for it to set before proceeding to the examination of the membrane), the branched cells will all draw in their processes, and appear in his preparation as round cells. I give this case as an example of the employment of a fixing agent for rapidly killing and hardening protoplasm without change of form; I will now give one to illustrate the usefulness of such an agent for rapidly hardening and fixing in their true form structures not protoplasmic. If a portion of living retina be placed in aqueous humour, serum, or other so-called "indifferent" medium, or in any of the media used for permanent preservation, it will be found that the rods and cones will not preserve the appearance they have during life for more than a very short time; after a few minutes a series of changes begins to take place, by which the outer ends of both rods and cones become split into discs, and finally disintegrate so as to be altogether unrecognisable, even if not totally destroyed. Further, in an equally short time the nerve-fibres become varicose, and appear to be thickly studded with spindle-shaped knots; and other post-mortem changes rapidly occur. If, however, a fresh piece of retina be treated with a strong solution of osmic acid, the whole of the rods and cones will be found perfectly preserved after twenty-four hours' time, and the nerve-fibres will be found not to be varicose. After this preliminary hardening, portions of the retina may be treated with water (which would be ruinous to the structures of a fresh retina), they may even remain in water for days without harm; they may be stained, acidified, hardened, imbedded, cut into sections, and mounted in either aqueous or resinous media without suffering.

For the study of the intimate processes of segmentation of ova, and division of cells generally, it is evident that the employment of fixing agents is a necessity; for it is a principal object of such study to seize upon particular moments of very transient phenomena, to investigate structural arrangements that exist only during a fleeting instant of cell-life, and that vanish without leaving a trace behind if the cells are allowed to die off slowly. The researches of Fol on the phenomena attending the impregnation of the ovum, and of Flemming and Strasburger on the division of cells and nuclei, could not have been carried out without some means of quickly rendering permanent the transient states it was desired to study.

Fixing agents, then, afford a means of preserving a record of moments of vital activity that would otherwise be inaccessible to observation. They also afford a means of seizing on transient states of functional activity, and preserving them for leisurely study. Ranvier wished to investigate the relations of the different parts of muscle substance in the different stages of contraction and relaxation of the muscle. Study of the living muscle would not suffice, as it is impossible to follow with the eye the rapid changes by which a muscle passes from the state of contraction to that of relaxation or the reverse. By the injection of a drop of osmic acid, muscular tissue is instantly fixed in the form it has when the acid comes into contact with it, and specimens of muscular fibre in all of its physiological states may be obtained and preserved as records of moments whose duration is measured by fractions of a second.

The processes that have been imagined for the preparation and permanent preservation of protozoa form another case in point. By simply fixing the animals on the slide by means of picric acid or osmium they may be stained, cleared, and mounted either in an aqueous medium, or in balsam, as successfully as the tougher tissues of higher organisms. To attempt to mount them in any medium without first subjecting them to that preliminary hardening brought about by the use of fixing agents would be hopeless. Lastly, I may give an example of the usefulness of fixation in cases in which it is desired to prepare contractile organisms in their natural extended state. Suppose you wish to mount a hydra with the tentacles extended, it is obvious that the animal must be killed with the utmost rapidity or the tentacles will be withdrawn before death, and you will have no means of getting them extended afterwards. If the animal be placed in a cell with a few drops of water, and if as soon as the tentacles are fully extended you add one drop of 2 per cent. solution of osmic acid the hydra will be killed with such lightning speed that not a tentacle will be retracted (Ranvier).

In blood we have an example of a tissue of which it is simply impossible to obtain permanent preparations well preserved without previous fixing. But if blood be treated, as a first step in the process of preparation, with picric acid or corrosive sublimate, it may be stained and mounted in some preservative medium with such success as to be available for the most minute study. And the same is true in general of other tissues. "Fresh tissue-elements or portions of tissue cannot be kept as preparations without undergoing modifications, such that after a few days their whole character is changed. Certain liquids have for a long time been recommended as having the property of preserving indefinitely tissues that are put into them in the fresh state; but in reality none of these liquids has such a property. It is possible to obtain permanent preparations of the most delicate organic elements; but in order to attain that end it is necessary to employ not one single preservative liquid, but a series of reagents, of which some serve to fix the elements in their form and render them more or less unchangeable, others to stain them, and others, finally, to preserve them from ulterior changes" (Ranvier).

5. A fixing agent should possess the following qualities: It must kill quickly, without exerting any injurious chemical action on the tissues; it must not be dehydratant, or it will cause shrinking; it must not interfere with subsequent processes, as by dyeing the tissues, or by preventing them from staining well, or by making them brittle. There is, I believe, only one reagent that is free from all these defects, viz. vapour of osmium; choice must be made amongst the others, whenever it is necessary to employ one of these, of that which has the least defects for the particular case.

Fixing agents may be conveniently grouped into two classes: those which do not seriously interfere with subsequent staining by carmine, and those which do. In the former class we have—

Alcohol.
Osmium vapour.
Corrosive sublimate.
Picric acid (either pure or in the form of Kleinenberg's fluid).
Merkel's solution.
Nitric acid.
Silver nitrate (in *weak* solutions).

In the latter class we have—

Chromic acid.
Chromate of ammonia.
Potassium bichromate.

Palladium chloride.
Silver nitrate (in *strong* solutions).
Osmic acid in solution.
Gold chloride.
Perchloride of iron.

It is not intended to be asserted that the quality of the carmine stain is necessarily seriously impaired by the use of these fixatives, but merely that they make staining uncertain, and sometimes, as in the case of chromic acid, so difficult of attainment by carmine or hæmatoxylin that the operator is driven to one of the anilin colours. Osmic acid comes into this category on account of its tendency to overblacken, and of the obstinacy with which it remains fixed in the tissues when used in solution.

It may be stated that, *as a general rule*, the reagents that best *preserve the forms* of cells are—

Chromic acid.
Osmic acid.
Picric acid.
Gold chloride.

The truest appearances are in most cases obtained by chromic or osmic acid; osmic acid will probably be preferred in the case of small organisms or portions of tissue on account of the great rapidity and energy of its action; but it has the great defect of possessing a very feeble penetrating power. Picric acid has very great penetrating power, and gives excellent results, but requires time for its removal from the tissues. Gold chloride is uncontrollable in its action.

For these and other reasons it will often be advisable to select one of the remaining reagents. One-third alcohol is somewhat feeble in its action, but is, on the whole, an admirable reagent for fixing, and has the great advantage of being the pleasantest of all to work with. Silver nitrate is useful for the study of superficial structures (epithelia), and nitric acid and corrosive sublimate are found to give admirable results in many cases. Bichromate of potash is a very useful reagent of general applicability.

The following are the points that it is most important to bear in mind:

Osmic acid must not be used with impermeable tissues.

Kleinenberg's solution is less adapted for the tissues of vertebrates than for those of invertebrates, on account of its causing connective tissue to swell.

Chromic acid is a very safe reagent, if not used in too strong solutions, and may always be used in case of doubt.

In the case of marine organisms it may be stated as a general rule that their tissues are more refractory to the action of reagents than are the tissues of corresponding fresh-water or terrestrial forms, and fixing solutions should in consequence be stronger (about two to three times stronger, according to Langerhans).

6. Heat as a Fixing Agent.—In some cases the simple application of heat is not only a good means of fixing, but the very best. Ova of some Arachnida, of Pycnogonida, ova and larva of some Bryozoa, are better fixed by this means than by any other. It will probably be found that this method is very helpful in the case of minute structures enclosed in very impervious cases of chitin or other impervious material; such cases or capsules obviously opposing but an inappreciable resistance to the passage of heat waves. The method consists simply in heating the objects to the temperature necessary to kill the cells and coagulate their contents. This is done by heating them in water in a watch-glass. It is, of course, better if practicable to throw them at once into water previously heated to the required temperature. I am not able to state precisely what that temperature should be; it appears, however, that a few seconds' immersion in boiling water is not hurtful even to delicate tissues.

The method has the great advantage of allowing of good subsequent staining, and of hindering less than any other the application of chemical tests.

The action of all the usual fixing agents (osmic acid, chromic acid, alcohol) is intensified and made more rapid by employing the solutions hot. Alcohol may sometimes be used boiling with advantage. Mayer finds that some *Tracheata* can only be satisfactorily fixed by means of boiling absolute alcohol.

7. Alcohol.—One-third Alcohol.—Alcohol is most usually employed for fixing purposes diluted to a strength of 33·3 per cent. This formula, which has now become classic, is due to Ranvier, who was the first to point out its wide applicability, and the excellence of the results obtained by it. Alcohol of the strength of 33·3 per cent. is known in France as "*Alcohol au tiers*," which is the name given to it by Ranvier himself; in Germany as "*Drittelalcohol*" or "*Ranviersche alcohol dilutus;*" in Italy, as "*alcool al terzo.*" I propose to speak of it as "*one-third alcohol.*"

8. One-fourth and one-fifth Alcohol.—For some purposes (*e.g.* retina) it has been found that still weaker alcohols give better results, viz. 25 per cent., or even 20 per cent.; but the strength of 33 per cent. is certainly the most generally useful.

Tissues should remain in these alcohols, as a general rule, for at least twenty-four hours; they may be stained (with picro-carmine, or, better) with some alcoholic staining medium. They must never be treated with water, or any aqueous liquid, except picro-carmine or alum-carmine, or methyl green.

9. Strong Alcohol.—Stronger alcohols are employed with advantage in some few cases; thus, for the study of voluntary muscle, 50 per cent. and 100 per cent.; for Spongida, and larvæ of Spongida, absolute alcohol often gives better results than any other reagent; it is recommended for the preparation of Magelona (Vermes).

10. Absolute Alcohol (*as a fixing agent*[1]).—Mayer finds that boiling absolute alcohol is often the only means of killing certain Arthropoda rapidly enough to avoid maceration brought about by the slowness of penetration of common cold alcohol (especially in the case of Tracheata).

11. Acidulated Alcohol (*Paul Mayer's formula*[1]).—To 97 vols. of 90 per cent. alcohol, in which is dissolved a small quantity of picric acid, add 3 vols. pure hydrochloric acid. Leave the specimens in the mixture only just long enough to ensure that they are thoroughly penetrated by it. Wash out with 90 per cent. alcohol, the disappearance of the yellow stain of the picric acid being a sign that all the acid is removed.

The use of this mixture is for the preparation of coarse objects it is intended to preserve in alcohol. The object of the acid is to prevent both that glueing together of organs by the perivisceral liquid, which is often brought about by the coagulating action of pure alcohol, and the precipitation on the surface of organs of the salts contained in sea-water, which is a hindrance not only to the penetration of the alcohol, but also to subsequent staining.

Whitman[2] states that "acid alcohol as above prepared loses its original qualities after standing some time, as ether compounds are gradually formed at the expense of the acid." He also states that 70 per cent. alcohol may be taken instead of 90 per cent., for washing out.

12. Osmic Acid.—Osmic acid is best employed in the form of vapour, and its employment in this form is indicated in all cases in which it is possible to expose the tissues directly to the action of the vapour. The tissues are pinned out on a cork which must fit well into a wide-mouthed bottle in which is contained a little solid osmic acid (or a small quantity of 1 per cent. solution will do). They remain there until they begin to turn brown. There is no need to wash them out before staining. Picro-carmine is a good stain to follow this process, which is certainly, where practicable, the most elegant that has hitherto been imagined.

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[1] 'Mitth. Zool. Stat. Neapel,' ii (1881), p. 7.

[2] 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 870.

When employed in aqueous solutions, osmic acid is used in strengths varying from $\frac{1}{20}$ per cent. to 2 per cent. Solutions of $\frac{1}{2}$ per cent. to 1 per cent. have been very largely used, but the tendency of modern practice seems to be towards weaker solutions and longer immersion. For Infusoria $\frac{1}{2}$ per cent. for a few seconds; for Porifera $\frac{1}{20}$ to $\frac{1}{10}$ per cent. for some hours; for Mollusca 1 to 2 per cent. for twenty-four hours; for epithelia $\frac{1}{10}$ to $\frac{1}{2}$ per cent. for an hour or two; for meroblastic ova $\frac{1}{10}$ per cent. for twenty-four hours; for medullated nerve-fibre $\frac{1}{10}$ to 1 per cent. for from twenty minutes to two hours; for tactile corpuscles $\frac{1}{3}$ to 1 per cent. twenty-four hours; for retina $\frac{1}{4}$ to 2 per cent. for from ten minutes to twenty-four hours; for nuclei $\frac{1}{10}$ to 2 per cent. for two or three hours. Such figures as these will serve to give a general idea of the practice, whilst more precise instructions will be given when dealing with the tissues in detail.

Osmic acid stains all fatty structures perfectly and opaquely black; it must therefore be avoided for tissues in which much fat is present.

Osmic acid must be well washed out before proceeding to any further steps in preparation; water may be used or glycerin. Notwithstanding the greatest care in soaking, it frequently happens that some of the acid remains in the tissues, and causes them to over-blacken in time. To obviate this it is necessary to wash them out in ammonia-carmine or picro-carmine, or to soak them for twenty-four hours in Müller's solution or in 0·5 per cent. solution of chromic acid, or in Merkel's solution, or in a weak solution of ferrocyanide of potassium or cyanide of potassium, or to bleach them (*see* BLEACHING, No. 476, *et seq.*). All solutions of osmic acid must be kept protected from the light even during the immersion of tissues. If the immersion is to be a long one the tissues must be placed with the solution in well-closed vessels, as osmium is very volatile.

Great stress is laid by authors on the fact that the vapour of osmium is very irritating to mucous tissues. It is said that the slightest exposure to it is sufficient to give rise to serious catarrh, irritation of the bronchial tubes, laryngeal catarrh, conjunctivitis, &c. I think these statements greatly exaggerated. I have frequently dissected for an hour at a time over a watch-glass containing 1 per cent. osmic acid solution and placed on the stage of the dissecting microscope, and that without experiencing any evil effects more serious than a disagreeable perception of the pungency and chokiness of the atmosphere. A good draught should of course be kept up by means of an open window or other arrangement. Stock solutions must be kept in stoppered bottles, and the bottles ought to be furnished with pipette stoppers or some contrivance by which a small quantity of the solution can be obtained when wanted without risk of portions of organic dust falling into the bottle.

13. Chromic and Osmic Acid Fixing Mixture (*Max Flesch's formula*[1]).—This mixture (osmium 0·10, chromic acid 0·25, water 100·0), originally introduced for the preparation of the auditory organ of vertebrates, is of general application. It does not require to be kept in the dark. Objects may remain in it for twenty-four to thirty-six hours without risk of the osmic acid over-blackening them. Flemming found it to preserve nuclear figures well; but the preparations are pale, and difficult to stain well. He finds that the action of the mixture is improved (for nuclear figures) by the addition of acetic, formic, or other acid. This addition brings out the figures more sharply, and has the further advantage of allowing of a sharper stain with hæmatoxylin, picro-carmine, or gentian violet. He recommends the following formula:

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[1] 'Arch. Mik. Anat.,' xvi (1878), p. 300.

14. Chromo-aceto-osmic Acid Fixing Mixture (*Flemming's formula*[1]).

Chromic acid	0·25 per cent.	In water.
Osmic acid	0·1 per cent.	
Glacial acetic acid	0·1 per cent.	

The best results (as regards faithfulness of fixation) are obtained with this mixture when it is allowed to act for only a short time (about half an hour).

15. Chromo-acetic Acid Fixing Mixture (*Flemming's formula*[2]).

Chromic acid	0·2 to 0·25 per cent.	In water.
Acetic acid ca.	0·1 per cent.	

Flemming finds this, followed by hæmatoxylin staining, to be the best means for demonstrating the achromatic figures. The preparations do *not* stain well with safranin or other anilins (p. 382).

16. Chromic Acid.—Chromic acid is employed in solution either in water or in alcohol.

The most usual strengths in which it is employed in aqueous solution are from 0·1 to 1·0 per cent. for a period of immersion of a few hours (structure of cells and ova). For nerve-tissues weaker solutions are taken, $\frac{1}{30}$th to $\frac{1}{8}$th per cent. for a few hours. Stronger solutions, such as 5 per cent., should only be allowed to act for a few seconds.

The objects should be washed out with water before passing into alcohol or staining fluids. Long washing in water is necessary to prepare them for staining, except an anilin stain be used.

Tissues that have been fixed in chromic acid may be stained in aqueous solutions if desired, as water does not appear to have an injurious effect on them; the acid appears to enter into some chemical combination with the elements of the tissues, forming with them a compound that is not affected either physically or chemically by water. The best stain to follow chromic acid is hæmatoxylin or safranin. But the previous washing out with water must be very thorough if good results are to be ensured; it may take days.

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[1] 'Zellsubstanz, Kern und Zelltheilung,' 1882, p. 381.

[2] Ibid., p. 382.

Chromic acid crystals are very deliquescent, and it is therefore well to keep the acid in stock in the shape of a 1 per cent. solution, which must be kept in a stoppered bottle in the dark, and care must be taken not to allow it to be contaminated by organic matter.

17. Chromic Acid and Platinum Chloride (*Merkel's solution*[1]).—Equal volumes of 1·400 solution of chromic acid and 1·400 solution of platinum chloride. Objects should remain in it for several hours, as it does not harden very rapidly. After washing out with alcohol, objects stain excellently, notwithstanding the admixture of chromic acid.

18. Nitric and Chromic Acid Fixing Solution (*Perenyi's formula*[2]).

4 parts 10 per cent. nitric acid.
3 parts alcohol.
3 parts 0·5 per cent. chromic acid.

These are mixed, and after a short time give a fine violet-coloured solution.

The objects (ova) are immersed for four to five hours, and then passed through 70 per cent. alcohol (twenty-four hours), strong alcohol (some days), absolute alcohol (four to five days). They are then fit for cutting. The advantage of the process is stated to be that segmentation spheres and nuclei are perfectly fixed, the ova do not become porous, and cut like cartilage.

Another advantage is that the fixing solution may be combined with a stain. (In this case the albuminous envelopes of the ova must be carefully removed, otherwise the stain will not penetrate.)

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[1] Merkel, 'Ueber die Macua lutea,' &c., 1870, p. 19; 'Mitth. Zool. Stat. Neapel,' ii (1881), p. 11.

[2] 'Zool. Anzeig.,' v (1882), p. 459.

Some stains, such as fuchsin or anilin red, may be dissolved directly in the fixing solution. Others, such as eosin, purpurin, anilin violet, must first be "dissolved in three parts of alcohol, and then shaken into the liquid."

Picro-carmine and borax-carmine may be added to the liquid, but they give rise to a precipitate, which must be removed by filtration before using. After staining (in this case) the ova are passed through 50 per cent. alcohol (five hours), common alcohol (ten hours), and then into absolute alcohol.

The ova are cut with alcohol and cleared with clove oil. Unstained ova may be stained after cutting by means of clove oil coloured with alcoholic solution of eosin or safranin. This is done by treating the sections on the slide for five or ten minutes with a drop or two of the coloured clove oil.

19. Chromic Acid and Spirit.—A mixture of two parts of $\frac{1}{6}$th per cent. chromic acid solution with one part of methylated spirit was much used by Klein in his investigations into the structure of cells and nuclei, and found to give better results than the ordinary reagents (including even osmic acid). Hæmatoxylin was used for staining.

20. Potassium Bichromate.—Used in solutions of from 1 per cent. to 2 per cent., or sometimes more, for most classes of objects. Flemming objects to this reagent for the study of nuclei, as not preserving nuclear figures in their true form. It is less of a hindrance to future staining than chromic acid. Wash out with water or alcohol. Altmann strongly recommends the use of a 2 per cent. solution, containing a little free chromic acid, and cooled to zero, followed by washing out in strong alcohol. The cooling of the liquid serves to stop instantly all molecular processes; and the slowly-acting mixture has time to complete the fixing.

21. Ammonium Chromate.—Appears to be generally used in 5 per cent. solution, for twenty-four hours. Wash out in water and stain in picro-carmine. This salt, like the preceding, is said by Flemming to distort nuclear figures, but many other workers consider it a very useful reagent.

22. Picric Acid.—Picric acid should always be employed in the form of a *strong* solution.[1] The saturated solution is the one most employed. Objects should remain in it for from a few seconds to twenty-four hours, according to their size. For Infusoria, one to at most two minutes will suffice; whilst objects of a thickness of several millimètres require from three to six hours' immersion.

Picric acid should *always be washed out with alcohol*, as water is hurtful to tissues that have been prepared in it. For the same reason, during all remaining stages of treatment, water should be avoided; staining should be performed by means of alcoholic solutions, the only exception to this rule being in favour of picro-carmine, which, probably on account of the picric acid contained in it, does not appear to exert so injurious an influence as other aqueous stains. It is one of the advantages of picric acid that, by sufficiently prolonged soaking, it can with certainty be entirely removed from any tissue by means of alcohol. Chromic acid, being combined with the elements of the tissues, cannot be so removed.

Tissues fixed in picric acid can, after removal of the acid by soaking, be perfectly stained in any stain. Mayer's cochineal, alcoholic borax-carmine, Kleinenberg's hæmatoxylin, Grenacher's alcoholic carmine, may be recommended.

The most important property of picric acid is its great penetration. This renders it peculiarly suitable for the preparation of chitinous structures. For such objects, alcohol of 70 per cent. to 90 per cent. should be taken for washing out, and staining should be done by means of Mayer's cochineal or Kleinenberg's hæmatoxylin.

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[1] That is to say, strong solutions must always be employed when it is desired to make sections, or other preparations of tissues with the elements *in situ*, as weak solutions macerate; but for dissociation-preparations, or the fixation of isolated cells, weak solutions may be taken. Flemming finds that the fixation of nuclear figures is equally good with strong or weak solutions.

In very many cases it is advantageous to employ picric acid in the manner suggested by Kleinenberg (see below), that is, in combination with sulphuric acid, nitric acid, or hydrochloric acid (*see* PICRO-SULPHURIC ACID, PICRO-NITRIC ACID, PICRO-HYDROCHLORIC ACID and the directions there given).

For the reasons stated below, picro-sulphuric acid is not, as a general rule, suitable for tissues of vertebrates.

23. Picro-sulphuric Acid (*Kleinenberg's formula*[1]).—"Prepare a saturated solution of picric acid in distilled water, and to a hundred volumes of this add two volumes of concentrated sulphuric acid; all the picric acid which is precipitated must be removed by filtration. One volume of the liquid obtained in this manner is to be diluted with three volumes of water, and, finally, as much pure kreasote must be added as will mix."

The addition of kreasote is intended to obviate a drawback that picro-sulphuric acid shares with osmic acid, viz., that of occasionally producing swellings in primitive blastomeres. (Mayer finds that kreasote produces no perceptible difference in the result, and has therefore abandoned it.)

"The object to be preserved should remain in this liquid for three, four, or more hours; then it should be transferred, in order to harden it and remove the acid, into 70 per cent. alcohol, where it is to remain five or six hours. From this it is to be removed into 90 per cent. alcohol, which is to be changed until the yellow tint has either disappeared or greatly diminished."

24. Picro-sulphuric Acid (*Mayer's formula*[2]).—A great deal of picric acid is precipitated by the sulphuric acid and wasted by Kleinenberg's mode of preparation. Mayer therefore now prepares the fluid as follows: distilled water, 100 vols.; sulphuric acid, 2 vols.; picric acid, as much as will dissolve. Filter and dilute with three vols. of water, except for Arthropoda, for which the fluid is used undiluted. The kreasote is omitted as being useless.

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[1] 'Quart. Journ. Mic. Sci.,' April, 1879, p. 208.

[2] 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 867.

Mayer directs that as the fluid does not diffuse very rapidly through thick chitin, the larger Arthropoda, such as Insecta, and the larger Isopoda, should be opened with scissors, and the body cavity at once filled with the solution by means of a pipette. A large quantity of the solution should be employed in all cases, and it should be changed as often as any cloudiness arises in it, and only when the fluid remains perfectly clear should objects be allowed to remain in the same change for any considerable time. This is a point that it is very important to attend to.

Washing out is done with alcohol of 70 per cent. *Warm* alcohol extracts the acid much more quickly than cold, with which *weeks* may be required to fully remove the acid from chitinous structures. I call attention here to what was said as to washing out under the head of *picric acid,* viz. that washing out must *never be done with water.* This is a most important point, and one that is not sufficiently attended to. (In a lately-published technical handbook, the student is directed to wash out picric acid preparations with liberal water!)

The advantages of picro-sulphuric acid as a fixing agent are, that it kills tissues very rapidly, that it has great penetrating power, that it can be totally soaked out of the structures with alcohol, leaving them in a good condition for staining, and, in the case of marine organisms, that it effectually removes the different salts of sea-water that are present in them.

It has some disadvantages. For vertebrata it should be used with caution, on account of the swelling caused by sulphuric acid in connective tissue. In parasitic Crustacea it also produces swelling and maceration and should be avoided (as was found by Fraisse, "Entoniscus Cavalini," n. sp., 'Arbeiten Zool. Zoot. Inst., Wurzburg,' 1877—78, iv, p. 383). Notwithstanding this, it is, however, according to Emery, very suitable for fishes, and for embryos of vertebrates generally, provided they are not allowed to remain in it more than three or four hours. For structures that contain much lime it is not to be recommended, for it dissolves the lime and throws it down as crystals of gypsum in the tissues. For such structures the picro-nitric or picro-hydrochloric acid is to be preferred.

25. Picro-nitric Acid (*Mayer's formula*[1]).—Prepared in the same way as picro-sulphuric acid except that instead of 2 vols. sulphuric acid you take 5 vols. pure nitric acid (of 25 per cent. N_2O_5). Mayer now dissolves the picric acid in the nitric acid water, so that the formula runs :

Water	100 vols.
Nitric acid (of 25 per cent. N_2O_5)	5 „
Picric acid, as much as will dissolve.	

The fluid is used undiluted.

The properties of this fluid are very similar to those of picro-sulphuric acid, with the advantage of avoiding the formation of gypsum crystals, and the disadvantage that it is much more difficult to soak out of the tissues. "Mayer recommends it strongly, and states that with eggs containing a large amount of yolk material, like those of Palinurus, it gives better results than nitric, picric, or picro-sulphuric acid."

26. Picro-hydrochloric Acid (*Mayer's formula*[2]).—Prepared in the same way as picro-sulphuric acid, except that instead of 2 vols. sulphuric acid you take "8 vols. of pure hydrochloric acid of 25 per cent. HCl." Mayer now dissolves the picric acid in the hydrochloric acid water, so that the formula runs—

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[1] 'Mitth. Zool. Stat. Neapel,' 1881, p. 5; 'Journ. Roy. Mic. Soc.' (N.S.), ii, 1882, p. 868.

[2] Ibid.

Water	100 vols.
Hydrochloric acid (of 25 per cent. HCl)	8 „
Picric acid, as much as will dissolve.	

The fluid is used undiluted.

The properties of this fluid are similar to those of picro-nitric acid.

Decalcification with picro-nitric or picro-hydrochloric acid.—The reader will perhaps reflect that the two last fluids appear likely to be very useful for decalcifications. Mayer points out that the action is very rapid, and that the copiously-evolved CO_2 often produces, mechanically, lesions in tissues; so that in many cases in which calcareous structures are concerned chromic acid is to be preferred, the more so as it more effectually hinders any *collapsing* of the structures that might result from the withdrawal of their supporting calcareous elements.

27. Nitric Acid.—Nitric acid has of late come into favour as a fixing medium for several classes of objects, but chiefly for cytological and embryological investigations. A strength of 5 per cent., with a duration of about thirty minutes (for small objects), may be recommended for all but the most delicate structures.

Flemming's formula.—Flemming used solutions as strong as 40 per cent. for the study of Karyokinesis in the ovum of Echinodermata.

Altmann's formula.—For the embryology of Vertebrata Altmann obtained the best results from solutions of 3 per cent. to 3½ per cent. (having a sp. gr. of about 1·02), which were allowed to act for a quarter to half an hour on small blastoderms, and for two to four hours on larger ones.

Uskoff's formula.—Uskoff recommends 5 per cent. solutions for from ten to thirty minutes. Wolff 10 per cent. (for the ovum of the fowl).

In all cases wash out, before staining, with strong alcohol.

Nitric acid is penetrating, fixes well, and allows of good staining. Some very delicate structures (cilia) appear to be lost in the present mode of employing it. It will probably came into much more general use.

28. Dilute Nitric Acid (*Altmann's methods*[1]).—(Of general histological application, but most specially referring to embryology.)

Fixing.—Dilute nitric acid (containing from 3 to 3½ per cent. pure acid). Such a solution has a sp. gr. of about 1·02; an areometer may conveniently be used to determine the concentration of the solution. Stronger solutions have been used, but do not give such good final results.

His[2] recommended a 10 per cent. solution. Altmann tried it, but found he could not demonstrate the nuclear figures. He considers that the strong solutions coagulate the soluble albuminoids of the tissues *too strongly*, which is a hindrance to the optical differentiation of structure. Flemming writes to Altmann that he employs solutions of 40 to 50 per cent. for the ova of invertebrates. This of course has the advantage of a very rapid fixing action.

The embryos, or other objects, are to be put *fresh* into the solution; it is useful, though not necessary, to employ a liquid cooled to zero; the cold stops all molecular processes, and the acid has time to fully complete the fixing process.

The objects must not be left too long in the liquid; for blastoderms and small embryos a quarter to half an hour is enough, for larger ones two to four hours. Only small pieces of tissues other than embryonic should be employed.

I understand the author to say that he then washes out the acid, and completes the hardening in strong alcohol. He points out that the process does not afford a true hardening such as is obtained by the use of chromic acid; but then he considers that by the use of paraffin imbedding such strictly so-called hardening is superfluous. With his modification of His's microtome he can cut such preparations to $\frac{1}{100}$ mm.

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[1] 'Arch. Anat. u. Phys.,' 1881, p. 219.

[2] Ibid., 1877. p. 115.

Before imbedding, the objects are stained *in toto*, slowly, with dilute hæmatoxylin. By moderately staining (either before or after the hæmatoxylin) with eosin, good double stains of nuclear figures are obtained, the chromatin structures taking up the blue colour.

For mounting; Xylol balsam.—Xylol is used instead of turpentine for dissolving away the paraffin from the sections; it evaporates quicker and more cleanly (Gaule).

The great advantages of this method are the perfection with which it fixes nuclear figures, and the perfection and readiness with which they stain (nitric acid being easily washed out by alcohol, and leaving no yellow stain behind, as chromic and picric acid mostly do). Altmann considers that nitric acid differentiates the structural elements of tissues more sharply from the surrounding dissolved albuminoids than osmic acid does. It is applicable to the most delicate objects of histology, particularly (with certain precautions, to be elsewhere detailed) to the retina. In a word, it is the most trustworthy fixing agent for protoplasm. A secondary point is that is the best of decalcifying agents.

29. Corrosive Sublimate.—Corrosive sublimate has of late been found to be one of the best of fixatives in a great variety of cases. It kills and hardens cells with great rapidity, and does not hinder the usual staining processes. It may be used pure or in combination with other reagents, as in the formulæ of Lang.

When used pure a cold saturated aqueous solution is taken, and allowed to act on the tissues until they are well permeated with it; they are then put for twenty-four hours into weak alcohol, stained, and subsequently further hardened with stronger alcohol if further hardening be desirable. It is sometimes well to wash out with water for half an hour before passing into alcohol.

30. Corrosive Sublimate (*Lang's method*[1]), *first formula. For Planaria.*—Take—

Distilled water . .	100 parts by weight.
Chloride of sodium .	6 to 10 parts.
Acetic acid . . .	5 to 8 ,,
Bichloride of mercury .	3 to 12 ,,
(Alum	½).

The Planaria are to be placed on their backs and the mixture is to be poured over them. They die extended. After the lapse of half an hour they are brought into alcohol, first of 70 per cent., then of 90 per cent., then absolute, and in two days' time are sufficiently hardened.

Second formula.[2]—Make a concentrated solution of corrosive sublimate in picro-sulphuric acid, to which has been added 5 per cent. of acetic acid.

Third formula.[3]—Take a concentrated aqueous solution of corrosive sublimate.

31. Corrosive Sublimate Solutions.[4]—Any of these solutions may sometimes be used *hot*, with good results. They are suitable for *Hydroida*, *Corallida*, small *Ctenophora*, some *Gephyrea*, *Balanoglossus*, *Echinodermata*, *Sagitta*, many *Annelida*, *Rhabdocœla*, and especially for *Dendrocœla*, for which last they are the *only* methods that give good histological results. *Cestoda* and *Trematoda*, and larvæ of *Turbellaria* may be added to the list. Lately good results have been obtained with crustacea with thin integuments, such as *Sapphirina*, and other *Copepods*, and with larvæ of *Decapods*. Good results have not been obtained with *Arthropoda* in general.

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[1] 'Zool. Anzeiger,' 1878, i, p. 14.

[2] Ibid., 1879, ii, p. 46.

[3] Loc. cit

[4] 'Mitth. Zool. Stat. Neapel,' ii, 1881, p. 11.

I have found the aqueous solution of sublimate very useful for *Appendicularia*. The simple aqueous solution certainly gave me better results than the more complicated formulæ; and I believe that most workers have arrived at the same result.

Objects should be removed from the fixing solution as soon as they have become soaked with it.

It must be remembered that the solutions must not be touched with iron or steel, as these produce precipitates that may hurt the preparations. To manipulate the objects, wood, glass, or platinum may be used; for dissecting them, hedgehog spines, or quill-pens.

Preparations that have beeen fixed in sublimate should not be allowed to remain long in alcohol, which makes them brittle; but after at most a few days they should be stained and imbedded in paraffin, in which they may remain until cut.

32. Modification of Lang's Formula (Acid sublimate solution) (*Carnoy's formula*[1]).

Water	100 parts.
Acetic acid	5 ,,
Bichloride of mercury	5 ,,

33. Modification of Lang's Formula (Saline sublimate solution) (*Carnoy's formula*[2]).

Water	100 parts.
Sodium chloride	3 ,,
Bichloride of mercury	5 ,,

34. Pacini's Solutions; Ripart et Petit's Solution.—The mercurial fluids of Pacini and the cupric solution of Ripart and Petit form excellent fixing solutions for many small and delicate objects. The formulæ are given in the chapter on "Examination Media."

35. Perchloride of Iron fixing Solutions (*Fol's methods*[3]).—

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[1] 'La Biologie Cellulaire,' p. 95.

[2] Ib.

[3] 'Zeit. Wiss. Zool.,' xxxviii (1883), p. 491.

Alcoholic solutions of this salt give the best results. Fol recommends that the *Tinctura ferri perchloridi*, B. P., be taken and diluted with water to about 2 per cent. If, however, it be desired to fix the whole contents of a good-sized vessel of sea-water, a somewhat stronger solution may be poured into the vessel. In no case, however, should a concentrated solution be added to sea-water, as this produces voluminous precipitates which render the preparations quite useless. As soon as all the organisms have sunk to the bottom of the vessel, the water is poured off, and the organisms are washed with 70 per cent. alcohol. (In all cases, whether weak or strong solutions have been employed, the organisms should only be allowed to remain in them for a very short time.) If it be desired to remove the iron from the tissues, the preparations should now be washed with a change of 70 per cent. alcohol, to which one or two drops of hydrochloric acid have been added. They may then be stained with carmine, but it is not advantageous so to stain them, as the tissues take up too much colouring matter and retain it obstinately, so that carmine-staining can only be employed, with such preparations, in the case of extremely thin sections. A more useful stain is obtained by putting the objects for twenty-four hours into alcohol containing a trace of gallic acid (a drop or two of a 1 per cent. solution). After again washing with alcohol it will be found that nuclei are stained intensely dark brown, protoplasm light brown. This stain is permanent.

If it be desired to obtain good stains with carmine, hæmatoxylin, or the like, this may be done (Prof. Fol informs me) by using oxalic acid (saturated solution in dilute alcohol) for removing the iron, instead of HCl.

The fixing action of iron perchloride is extremely rapid. Fol found it was the the only reagent with which *Tintinnodea* could be usefully preserved. Larger pelagic animals such as *Medusæ*, *Ctenaphora*, *Salpæ*, *Heteropoda*, and the most delicate pelagic larvæ are alike perfectly preserved by this method, as regards both their general form and their histological detail.

For the fixing of the ova of *Ascidians*, Fol recommends a mixture of one part of the *Tinctura ferri perchloridi* with ten parts of 70 per cent. alcohol, instead of water ('Recueil Zoologique Suisse,' i, 1883, p. 121).

36. Palladium Chloride.—Palladium chloride has been recommended by experienced workers. It is used in solutions of 1·300, 1·600, or 1·800 strength, for from one to two minutes.

37. Gold Chloride.—When used for fixing (and not for the object of staining by impregnation) gold chloride is generally used in solution of $\frac{1}{2}$ per cent. strength, for a few minutes (30 at most). Weaker solutions ($\frac{1}{5}$th per cent.) or stronger (1 to 2 per cent.) may also be used. Wash out with water. Fixes well, but is uncertain in its action, and generally a hindrance to staining processes.

38. Silver Nitrate.—Silver nitrate is frequently used in the study of epithelia, not alone for the purpose of demonstrating the outlines of cells by staining the intercellular cement-substance, but also with the totally distinct object of rapidly fixing the cells. Solutions of from $\frac{1}{2}$ to 2 per cent. are employed and allowed to act for merely a few seconds. Solutions of only 3 to 1000 strength may be allowed to act for an hour. Wash out with water. Stain as desired. Weak solutions, rapidly applied, do not hinder subsequent staining; strong solutions do.

Silver nitrate sometimes gives very good results, but the conditions under which these results are obtained are not sufficiently understood for the operator to be able to produce them with certainty. For this purpose then, as for that of staining, nitrate of silver is at present a reagent too uncertain in its action to be generally recommendable.

39. Pyroligneous Acid.[1]—It is stated by an anonymous writer in the Berlin 'Zeitschrift. f. Mikroskopie' that pyroligneous acid instantly kills Infusoria, Rhizopods, Daphnia, Cyclops, Algæ, &c., without altering their form. The acid used is the *Acetum pyrolignosum rectificatum*. When it has become turbid it must be filtered before being used.

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[1] 'Journ. Roy. Mic. Soc.,' ii (1879), p. 180.

The objects may be stained by means of anilin colours dissolved in the acid.

Dissolve 1 part of anilin blue or diamond fuchsin in 200 parts distilled water; filter; and add 800 parts of pyroligneous acid.

The objects take some hours to stain. Apparently the author of this process mounts the objects in the solution with which they were fixed!

40. Acetic Acid. Formic Acid.—These two acids are useful and well-known fixatives of nuclei. Flemming, who has made a special investigation of their action, finds ('Zellsubstanz,' &c., p. 380) that the best strength is from 0·2 to 1 per cent. Strengths of 5 per cent. and more bring out the nuclein structures clearly at first, but after a time cause them to swell and become pale, which is not the case with the weaker strengths (ibid., p. 103).

41. Alum.—Alum has been used for fixing purposes, and may therefore be mentioned here. Although quite superseded for general work by other reagents, it may possibly still be found useful for certain special purposes. For instance, for the preservation of *Medusæ* the following process has been recommended (by Pagenstecher). Take two parts of common salt and one of alum, and make a strong solution. Throw the animals into it alive, and leave them there for twenty-four to forty-eight hours. Preserve in weak alcohol. A saturated solution of alum in sea-water preserves very well the forms of *Salpidæ*, *Medusæ*, *Ctenophora*, and other pelagic animals. It constitutes a preservative medium in which the objects may remain till wanted.

42. Iodine.—Iodine possesses considerable hardening properties, and a very high degree of penetration; and, in point of fact, iodized serum, which is generally employed as an "indifferent liquid," that is, one which is supposed to exert no action whatever on tissues, is, in reality, a feeble hardening agent, and forms a most admirable fixing agent for delicate tissues. It is so classed by Ranvier, *see* **Iodized Serum,** No. 447 (Macerating Agents).

43. Potassic Iodide (*Kent's method*[1]).—"Mr. W. S. Kent has found potassic iodide to act in a manner almost identical with osmic acid, and in some instances even more efficiently" (for fixing Infusoria). "Prepare a saturated solution of potassic iodide in distilled water, saturate this solution with iodine, filter, and dilute to a brown-sherry colour. A very small portion only of the fluid is to be added to that containing the Infusoria."

43a. Potassic Permanganate (*G. du Plessis' method*[2]).—According to the translator in 'Science Gossip,' from whom I quote, permanganate of potash "is especially good in histological researches, as it acts like osmic acid, burning up (*sic*) the protoplasm, bringing out the minutiæ, and showing the nuclei, outlines of cells, &c. It is used as a saturated solution in distilled or very pure spring water." . . . "The concentrated solution, of a lovely violet colour, kills small organisms at once, and then burns them" (*sic*). "They are left in it from thirty minutes to an hour, then withdrawn and placed in alcohol. . . . Beautiful results are thus obtained with Echinoderms, Zoophytes, worms, and marine Arthropoda. For delicate researches, especially in the ciliated Infusoria, it is better than osmic acid. . . ."

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[1] Kent, 'Manual of the Infusoria,' 1881, p. 114. 'Journ. Roy. Mic. Soc.' (N. S.), iii (1883), p. 730.

[2] 'Bull. Soc. Vaud. Sci. Nat.,' 2, sér. xv, pp. 278—280, 1878. 'Science Gossip' (date ?).

CHAPTER III.

THEORY OF STAINING.

44. The chief end for which colouring reagents are employed in zoo-histology is to obtain a *nuclear* stain of tissues, that is, a stain in which nuclei, or at most, the nuclei and their surrounding cell-protoplasm, are coloured, whilst the formed material of the tissues is left unstained. That is what the histologist wants in the great majority of cases. He wants either to differentiate the intimate structures of cells by means of a colour reaction, in order to study them for their own sake, or he wants to have the nuclei of tissues marked out by staining in the midst of the unstained formed material in such a way that they may form landmarks to catch the eye, which is then able to follow out with ease the contours and relations of the elements to which the nuclei belong; the extra-nuclear parts of these elements being expressly left unstained in order that as little light as possible may be absorbed in passing through the preparation. Diffuse stains, or those which stain formed material as well as protoplasm, are now more and more abandoned; for instance, eosin, which was once a favourite stain, is now but little used, on account of the incorrigible diffuseness with which it stains. Except for special purposes, such as the dyeing of thin membranes which, unstained, would be invisible, or for certain purely chemical ends, or for combination with a nuclear stain to make a double stain, diffusely-staining colouring agents are not employed.

As a general rule, one indeed to which it is difficult to find a plausible exception, all alkaline staining solutions should be avoided. Alkalies dissolve nuclein, or if they do not dissolve it when very dilute, swell and distort nuclear structures, and are frequently hurtful to formed material. Neutral or acid stains should alone be used, and it will probably be found that better preparations are obtained with acid solutions than with neutral ones. It is most important to work with acid stains in all cases in which it is desired to faithfully preserve *nuclein* formations (Carnoy, l. c., p. 211).

In order to obtain precise stains it is important to operate on tissues that have been carefully fixed; or, if fresh tissues be taken, that the staining solution should itself be a sufficient fixative. An important progress has been lately realized by the discovery that it is possible to combine the fixative action of osmic acid with some stains (*see* **Alum-carmine** and **Osmium and Methyl-green,** Carnoy's method) by adding a trace of the acid to the staining solution. It is to be hoped that the means of combining osmic acid with many other stains will be discovered, as the fixing action of osmic acid would be most desirable in cases in which staining is performed by means of *prolonged* immersion in an aqueous medium. Carnoy states that he employs osmium with almost all his liquids (picro-carmine is an exception, as osmic acid precipitates it). In the same way, acetic acid (which is a good fixative for nuclei) may be combined with many stains; for instance, with alum-carmine, methyl-green, safranin, gentian violet, Bismarck-brown, nigrosin, and others (Carnoy).

In the Introduction I have pointed out the desirability of employing alcoholic stains for objects that have been treated with alcohol; it will be sufficient here to again call attention to the superior penetrating power of alcoholic solutions. The histologist should never be without a good alcoholic stain.

The question of permanence of stains has some importance. Carmine is certainly, in my opinion, the colouring matter that can most be relied on in this respect. It is affirmed by a recent writer (A. C. Cole, in "Methods of Micr. Research," vii, 1884, p. 41, *cf.* 'Journ. Roy. Mic. Soc.' (N.S.), 1884, p. 310) that "no stain has been found to equal logwood for certainty and permanence of results," but he affirms also, that this permanence is only obtained with benzol-balsam mounts. Flemming[1] finds hæmatoxylin stains lose in sharpness after about a year, both in glycerin and in resinous media (Flemming does not state which resinous media he means, but it is probable he refers to dammar). Other writers assert the stain to be permanent both in glycerin and in dammar, provided certain precautions be taken, for which see Nos. 92 and 93.

We do not yet possess sufficient data to enable us to judge surely of the permanence of anilin stains. Flemming, writing in 1882 (l. c., p. 384) states that of all his preparations safranin and napthalin (dammar mounts) have kept the best, showing no change whatever since 1878. Chromic acid gentian-blue stains fade *a little* in the course of a year, but not so much as the hæmatoxylin stains. It is certainly premature to condemn anilin stains *en bloc* for a want of permanence that has not been proved against them.

The same writer (Cole) appears to assert that picro-carmine stains keep better in glycerin jelly than in any other medium; he says that "a preparation stained with picro-carminate of ammonia and mounted in good glycerin jelly is unchangeable." Doubtless picro-carmine stains are well preserved in glycerin jelly, but so they are in all the usual media; in acidulated glycerin (formic acid glycerin) they are still better preserved; and in dammar, balsam, or colophonium they, in common with other carmine stains, keep perfectly. And as for the unchangeableness of glycerin-jelly preparations, does Mr. Cole, who asserts that they "would last a thousand years, and be as perfect the last day as on the first," really mean such statements to be taken seriously?

The greatest difficulty in the technic of staining lies in the incompatibility of certain fixing agents with certain staining agents. Thus chromic acid and osmic acid, the two best fixing agents known, are to a great degree incompatible with staining by carmine, the most trustworthy of staining agents, often rendering the operation of staining with carmine so difficult that it is better to abandon it and employ some other stain. A few hints on this distressful matter may here be useful.

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[1] 'Zellsubstanz,' p. 384.

Hæmatoxylin is the best stain to use after chromic acid; but some anilins (safranin, magdala, dahlia, for instance) give good results when employed by the Hermann Böttcher process (*see* **Anilins**, No. 136).

Cochineal may also be used after chromic acid.

After osmic acid, picro-carmine or alum-carmine, or hæmatoxylin. But osmic acid preparations generally stain well only after *bleaching* (*see* BLEACHING).

All stains take well after fixation with alcohol or with corrosive sublimate, or with nitric acid.

It should be noted that, as found both by Flemming and by Mayer, objects that have been too long in alcohol, or that have been treated with very strong alcohol, no longer take a precise nuclear stain, either with anilins or with other colouring agents.

After fixation with any of the picric-acid fluids, and due washing out with alcohol, all objects stain well with any of the usual stains.

45. Carmine.—Carmine, which is probably the most valuable and certainly is the most widely employed of histological colouring agents, was first proposed as an aid in the examination of animal tissues by Gerlach in 1858, since which time, notwithstanding the discovery of numerous other substances that have proved most useful in many kinds of research, it has held its place, with a firmness that shows few signs of yielding, as the staining agent *par excellence*.

It is not a definite chemical substance. As is well known, it is prepared from the cochineal insect, *Coccus cacti*, by processes of which the details are trade secrets, and which may or may not vary in essential points. (Hence the complaints that the carmine of commerce is of varying quality, and hence probably the fact that so often meets the practical worker, that carmine solutions carefully prepared according to a given formula do not necessarily possess the properties assigned to them by the author of the formula. The operator should bear this in mind whilst studying these stains.) It is obtained by treating the *Coccus cacti* with salts of tin. "It consists of the colouring principle, carminic acid, together with coccin, stearin, olein, and other organic compounds, carbonate and phosphate of lime, phosphate of potash, and chloride of lime." It is freely soluble in ammonia, but the simple aqueous solution thus obtained is not stable. The causes of this instability have been studied by Betz of Kieff. He reports as follows concerning the precipitate obtained by exposing ammoniacal solutions of carmine to sunlight and air ('Arch. Mik. Anat.,' ix, 1873):

"The above-mentioned flocculent precipitate, being washed with water, is found to retain a portion of the colouring matter of the carmine. It gives the following reactions. Part of it is soluble in caustic potash. The solution is dark-red, and gives with acetic acid a flocculent precipitate which re-dissolves in concentrated acetic acid. This last solution gives no reaction with red prussiate of potash, but with the yellow prussiate it gives a precipitate. The precipitate thrown down by acetic acid from the potash solution contains no colouring matter. If to the potash solution be added sugar and sulphuric acid, a weak and transient violet colouration is produced at the surface of contact. On boiling the potash solution with nitrate of mercury, a violet colouration is obtained. The precipitate thrown down by acetic acid from the potash solution dissolves in boiling concentrated HCl without giving any colouration.

"The part of the original precipitate that is insoluble in caustic potash is also insoluble in concentrated acetic acid. With sugar and sulphuric acid it gives no violet reaction. It is only partially dissolved by boiling with HCl, and the solution is colourless. Treated with sulphate of copper it becomes bright red. Iodine stains it yellow, ammonia dissolves it."

It is concluded from these reactions, (1) That the ammoniacal solution of commercial carmine contains two distinct albuminoid substances, which under the influence of light and heat are in part decomposed, in part separated out from the solution; (2) that the staining properties of the solution depend on the presence of small traces of free ammonia (a proposition which appears to the present writer to be in disagreement with other facts); and (3) that the often-observed sudden appearance of fine granules in filtered carmine solutions depends on the separation from the solution of the albuminoid bodies, and not of hydrate of aluminium, as has been supposed by some. The reactions of the precipitates show that the part soluble in caustic potash corresponds to paralbumin, the insoluble part to ichtydin.

46. Carminic Acid.[1]—"G. Dimmock has often wondered why naturalists use carmine solutions in which water, with some caustic or destructive material added, is the principal solvent. Carmine of commerce, it is true, is not readily soluble even in water, until ammonia, borax, or some other aid to solution is added, but *carminic acid*, the basis of the colouring matter of carmine, has long been stated in the leading chemical dictionaries and handbooks to be readily soluble in water and in alcohol.

"The author employs a solution of 0·25 gr. carminic acid to 100 gr. of 80 per cent. alcohol, and leaves sections in the solution from two to five minutes.

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[1] 'Amer. Natural.,' xviii (1884), pp. 324—7. 'Journ. Roy. Mic. Soc.' (N. S.), iv (1884), pp. 471—474.

"An alcoholic ammonic carminate, or ammonia carmine, can be prepared at a moment's notice from alcoholic carminic acid by adding ammonia drop by drop and stirring until the entire solution changes from its bright red to purple red.

"Alcoholic carminic acid may be used as Grenacher's carmine solution is used to colour sections from which the colour is to be afterwards extracted by very dilute hydrochloric acid, leaving nuclei red. Another way to use carmine solutions, which is especially applicable to alcoholic carminic acid, is to precipitate the carmine in the tissues by some salt, the carminate of the base of which gives a desired colouration. For example, specimens hardened for a moment under the cover-glass with an alcoholic solution of corrosive sublimate (mercuric chloride) and after washing with alcohol, coloured in alcoholic carminic acid, take a fine colour of mercuric carminate. So, too, specimens coloured in alcoholic carminic acid can be changed by a few moments' treatment with a very dilute alcoholic solution of lead acetate or cobalt nitrate to a beautiful purple. Sometimes salts in the tissues of the animals change portions of the carminic acid to purple carminates, giving a double colouration without further treatment.

"Picric acid added to alcoholic carminic acid in extremely small quantities (best in a dilute alcoholic solution, testing the solution on specimens after each addition) makes a double alcoholic colouring fluid (a so-called picro-carmine). The author has been unable thus far to determine the proportion of picric acid required for this solution, having in every case added an excess. All different kinds of carmine solutions can be made from carminic acid with the advantage of having always uniform strength, of being definite mixtures, and of not spoiling as readily as those made from cochineal."

To these advantages may be added that carminic acid may be preserved dry without decomposition. The author notes that all alkaline solutions and nearly all metallic salts are incompatible reagents (with carminic acid); and that all acids are so with ammonic carminate.

47. Preparation of Carminic Acid; De la Rue's Method.—The following methods of preparation are given: "The first mode is that of De la Rue, which Watts ('Dict. Chem.,' 1, 1863, p. 804) gives as follows:—"To separate carminic acid, cochineal is exhausted with boiling water; the extract is precipitated by sub-acetate of lead slightly acidulated, care being taken not to add the lead solution in excess; the precipitate is washed with distilled water till the wash-water no longer gives a precipitate with a solution of mercuric chloride, then decomposed by sulphuretted hydrogen; the filtrate is evaporated to a syrupy consistence and dried over the water bath; and the dark purple product thus obtained is treated with alcohol, which extracts the carminic acid."

48. Schaller's Method.—The second mode is that of C. Schaller and is given by Watts ('Dict. Chem.,' 1st Suppl., 1872, p. 413) as follows:—"Schaller prepares this acid by precipitating the aqueous extract of cochineal with neutral lead acetate acidulated slightly with acetic acid; decomposing the washed precipitate with sulphuric acid; again precipitating the filtrate with lead acetate and decomposing the precipitate with hydrogen sulphide. The filtered solution is evaporated to dryness; the residue dissolved in absolute alcohol; the crystalline nodules of carminic acid obtained on leaving this solution to evaporate are freed from a yellow substance by washing with cold water, which dissolves only the carminic acid; and the residue left on evaporating the aqueous solution is re-crystallised from absolute alcohol or from ether."

"Schaller's mode of preparation gives purer carminic acid than De la Rue's, but either kind is sufficiently pure for histological purposes. The precipitation by lead acetate and the dissolving in alcohol free the carminic acid from animal impurities, and the consequence is a purer form of pigment.

than can be extracted by any process hitherto employed for the preparation of carmine for histological purposes." . . . .

It is not certain whether the stain of carminic acid is stable in glycerin. "Some preparations coloured in alcoholic carminic acid and then put up in glycerin lost their colour in a few months, while similar preparations mounted in Canada balsam retained their colour perfectly." But the author thinks this may have been due to impurity of the glycerin employed.

For another mode of preparation of carmine *see* **Picro-carmine**, Pergens' formula, No. 70.

49. Carmine Stains in General.—Carmine was at first employed in histology in the form of ammoniacal solutions; and various mixtures made on this principle are still used. It was, however, at length clearly seen that the use of the ammoniacal solutions had two serious disadvantages; firstly, the above-mentioned instability of the solutions, which from the very moment of their formation are engaged in a series of ill-understood chemical processes that makes it impossible for the anatomist to know whether at any moment his solution is in a state fit for producing a good stain; and, secondly, the fact that the free ammonia of the solutions has a highly injurious action on many delicate tissue elements. The diversity of the many formulæ for carmine staining fluids given below is in great part the result of attempts to find solutions that should be stable and should not contain free ammonia. Such are the neutralised ammoniacal solutions of Betz, Heidenhain, and Hoyer; such is the celebrated picro-carmine of Ranvier, and such are the alum-carmine and borax-carmine of Grenacher.

The especial value of carmine lies in its property of readily affording a nuclear or protoplasmic stain. This property was explained by Beale by means of the hypothesis that recently dead protoplasm has an acid reaction. "Hence if an alkaline solution of colouring matter from which the colour may be precipitated or fixed by an acid be caused to pass into it, the alkali is neutralised by the acid present, and the colour is retained. The tissue itself, or formed material, being ordinarily bathed with an alkaline fluid, does not take the colour."

That this explanation is not the right one will appear evident from a simple inspection of the following formulæ, amongst which will be found both neutral and acid fluids giving a nuclear stain.

The formulæ set out below are arranged according to the nature of the menstruum. This gives us two great groups: aqueous carmine solutions and alcoholic carmine solutions. Taking first the group of aqueous solutions I have arranged the formulæ comprised in it according to the reaction of the solutions. First come the alkaline ammoniacal solutions, then the neutralised ammoniacal solutions, then other neutral solutions, including alum and picro-carmine, and borax carmine. These last requiring a treatment of the tissues with an acid to fix the stain lead naturally to the last group, the acid stains. The alcoholic group is too small to require subdivision.

The following statements are, I believe, true of all carmine stains. Tissues to be stained must be freed from acids before being put into the staining fluid. Overstains may in all cases be washed out with weak HCl. (*e.g.* 0·1 per cent.). It if generally advisable to fix the stain in the tissues before mounting. In the case of balsam mounting this is sufficiently done by the action of the alcohol; in the case of an aqueous mount acetic or formic acid should be employed, and the best way of doing this is to let the mounting medium contain 1 per cent. of formic or acetic acid. Formic acid is to be preferred.

The beginner may wish for some hints as to which of these formulæ he should choose.

If his tissue has been prepared (*i.e.* fixed, hardened, and preserved) in alcohol he ought to choose one of the alcoholic solutions; or, if he take the less advisable course of using an aqueous one, then it should be picro-carmine or alum-carmine.

Amongst the aqueous stains, I consider that alum-carmine is the most precise and is the one that has the most differentiating power; by which is meant that by it different tissues are stained with varying degrees of intensity and with varying tints.

Picro-carmine is perhaps the least hurtful to tissues, but alum-carmine comes near to it in this respect.

Ammonia-carmine should only be employed in the case of tissues, which, having been thoroughly impregnated with one of the metallic hardening agents (osmium, chrome compounds, and the like), are not liable to suffer from the swelling and maceration that ammonia brings about in tissue not so prepared. As a general rule it should be carefully avoided. Remember that none of Grenacher's fluids can be used with calcareous structures that it is wished to preserve.

Wherever it is possible alcoholic solutions should be preferred; they are less hurtful to the tissues than aqueous solutions, more penetrating and generally more precise in their action. Grenacher's alcoholic borax-carmine may be recommended to the beginner as being the easiest of these stains to work with.

Lastly, it may be observed that in many cases in which such a stain is required, it will be found equally satisfactory in result and convenient in practice to have recourse to alcoholic cochineal.

CHAPTER IV.

CARMINE STAINS (AMMONIACAL).

50. Ammonia-carmine (*Beale's formula*[1]).

Carmine	10 grains.
Liquor Ammoniæ (fortissimus, B.P.)	½ drachm.
Price's glycerin	2 ounces.
Distilled water	2 ounces.
Alcohol	½ ounce.

The carmine, in small fragments, is to be dissolved in the ammonia, with the aid of heat. Boil for a few seconds, and let cool. Leave uncorked for at least an hour, or until the excess of ammonia has evaporated, as tested by the smell. Then add the glycerin, water, and alcohol, and filter, or allow to settle and decant. If after keeping for some months the carmine begins to precipitate, owing to the escape of ammonia, add one or two drops of liquor ammoniæ.

This fluid requires to be made stronger or weaker in particular cases, and great advantage sometimes results from diluting it with alcohol (to increase its penetration).

Like the following ammoniacal solutions of carmine, this is a nuclear stain, *when perfectly successful*. But it is very difficult to get perfect nuclear stains with it in the case of tissues that have been treated with chromic acid, &c. Tissues hardened in alcohol or picric acid stain well in it.

"The rapidity with which colouring takes place depends partly upon the character of the tissue, and partly upon the excess of ammonia present in the solution. If the solution be very alkaline, the colouring will be too intense, and much of the soft *tissue* or imperfectly-formed material around the germinal matter is destroyed by the action of the alkali. If, on the other hand, the reaction of the solution be neutral, the uniform staining of tissue and germinal matter may result. When the vessels are injected with the Prussian-blue fluid, the carmine fluid requires to be sufficiently alkaline, to neutralise the free acid present. The permeating power of the solution is easily increased by the addition of a little more water and alcohol. In some cases the fluid must be diluted with water, alcohol, or glycerin; and the observer must not hastily condemn the process, or conclude, as some have, that a particular form of germinal matter is not to be coloured, till he has given the plan a fair trial, and tried a few experiments."

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[1] 'How to Work, &c.,' p. 109, 4th ed.

The present writer, who has tried this fluid by using it daily for more than a year, feels constrained to disagree with Prof. Ranvier, who teaches that it "presents no real advantages, whilst it has the disadvantage of a tendency to diffusion of the colouring." He may be right in the latter remark; but it is self-evident that the excellent preservative solution afforded by this mixture of weak glycerin and alcohol, is a very "real advantage" both as regards the preservation of the tissues immersed in it, and the stability of the liquid itself. The diffuseness of the colouration may always be counteracted by washing in weak HCl.

51. Ammonia-carmine (*Beale's second formula*[1]).

Carmine	15 grains.
Liq. Amm. fortissmus . . .	½ drachm.
Price's glycerin	2 ounces.
Alcohol	6 drachms.

This fluid is specially designed for the purpose of staining by means of injection. Inject, and leave the preparation twenty-four hours. Then inject a little pure glycerin, to wash out the vessels; finally inject the Prussian-blue fluid ('How to Work, &c.,' p. 296). I have tried it for staining sections, and small portions of tissues, in the ordinary way; and find it answers well.

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[1] 'How to Work, &c.,' p. 304.

52. Ammonia-carmine (*Ranvier's formula*[1]).

Distilled water	100
Ammonia	1
Carmine	1

Rub up the carmine in a mortar with a little of the water; add the ammonia, and when the carmine is dissolved, add the rest of the water. If there remain an excess of ammonia, heat over a water-bath until precipitation of the carmine begins.

53. Ammonia-carmine (*Frey's formula*[2]).

Carmine	15 to 30 centigrammes.
Ammonia	quant. suff.
Water	30 grammes.

Dissolve, filter, and add—

Glycerin	30 grammes.
Strong alcohol	8 to 12 grammes.

More glycerin may be added if desired. Ranvier quotes this formula with approval. It is very difficult to see in what respects it differs from Beale's formula, which he quotes with disapproval.

54. Ammonia-carmine (*Huxley's and Martin's formula*[3]).

Carmine	2 grammes.
Strong solution of ammonia	4 c.c.
Distilled water	48 c.c.

Dissolve the carmine in the ammonia and water; leave in an unstoppered bottle until nearly all smell of ammonia has gone. Afterwards keep in a well-closed bottle. Dilute a small quantity with fifteen or twenty times its bulk of water, when required for use.

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[1] 'Traité Technique,' p. 97.

[2] 'Le Microscope,' p. 167.

[3] 'Practical Biology,' p. 268.

55. Ammonia-carmine (*Betz's formula*[1]).

Commercial carmine is rubbed up with a little water in a mortar until a thick syrupy mass is obtained; on to this, ammonia is poured, with continual stirring. The solution is diluted with a large quantity of water and filtered. The filtered solution is exposed to the sun in an uncorked vessel, which must be of *green* glass, until a dirty-red, flocculent precipitate appears; it is then filtered. The filtrate is again left to stand in the same conditions as before, and when the precipitate reappears, it is again filtered, and the filtrate again exposed. Generally no third precipitate appears; if it does, filter again. In either case, the preparation is now finished, and the solution is to be preserved for use in a corked vessel. It will keep for months. It sometimes happens that the solution whilst exposed to the sun acquires a bad smell, and becomes covered with a white flocculent membrane. This does not hinder the preparation, but, on the contrary, furthers it.

Half an hour, or at most an hour, suffices to stain sections. The first elements that stain are the granular mass of the grey matter, then nerve-cells, epithelium, and lastly, other structures. A nuclear stain. Permanent.

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[1] 'Arch. Mik. Anat.,' ix (1873), p. 112.

CHAPTER V.

NEUTRAL CARMINE, ALUM-CARMINE, AND PICRO-CARMINE.

56. Neutral Carmine (*Heidenhain's formula*[1]).—A carmine solution is prepared according to *Beale's formula* (No. 50), but with the omission of the alcohol. This is rendered *almost neutral,* either by cautious addition of dilute acetic acid or by driving off the free ammonia by warming on a water-bath. (The test for approximate neutrality is, that a watch-glassful of the solution, allowed to stand uncovered, should precipitate all its carmine, through evaporation of the ammonia, in the course of twenty-four hours.)

The method of using is as follows:—The sections are placed in a watch-glassful of the solution, and the watch-glass is placed in an air-tight vessel together with a second watch-glass which contains water having a trace of ammonia (just enough to be perceptible by the smell). This small quantity of ammonia, gradually evaporating and being absorbed by the staining solution, suffices to keep the carmine dissolved for twenty-four to twenty-eight hours, by which time the staining is generally complete. The sections are washed in common glycerin, then brought into a watch-glassful of concentrated glycerin and exposed for twenty-four hours in a closed vessel to the vapour of a small quantity of acetic acid. Mount in glycerin. The stain is elective for certain cells (of the peptic glands, *see* the article quoted); it is nuclear and permanent in glycerin. Should an overstain happen, it may be washed out by exposing the sections in glycerin to the action of vapour of ammonia.

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[1] 'Arch. Mik. Anat.,' vi (1870), p. 402.

Another method of employing this stain (for special purposes, for which *see* the article) is as follows:—A few drops of the carmine solution are added to a watch-glass of concentrated glycerin, so as to give a very light red colour. Such a mixture has no staining action by itself, but if sections be placed in it and exposed to the action of vapour of acetic acid, staining gradually occurs. This is the best method for differentiating the "Haupt-" and "Belegzellen" (in the rabbit). Overstains may be removed by exposing to the vapour of HCl.

57. Neutral Carmine (*Böhn's formula*[1]).—Three to 4 grms. carmine are rubbed up in a mortar with 200 grms. water, and ammonia is added drop by drop until the solution acquires a cherry-red colour; acetic acid is then added until the colour becomes of a sealing-wax red; and the solution is filtered. (If the colour is not intense enough, add before filtering two drops of ammonia, and leave in an open vessel until the smell of ammonia can no longer be perceived.)

Tissues should remain for twenty-four hours in the stain (or longer if they are more than 1 mm. thickness), after which it is desirable, in order to ensure a nuclear stain, to wash out with glycerin and water (equal parts) containing ½ per cent. of hydrochloric acid.

These directions apply to blastoderms.

58. Neutral Carmine (*Hoyer's formula*[2]).—"Dissolve 1 gr. of carmine in a mixture of 1—2 c.c. of strong liquor ammoniæ and 6—8 c.c. of water. Heat in a glass vessel on a sand bath until the excess of ammonia has evaporated. (So long as free ammonia is present *large* bubbles are formed in the fluid, and the latter shows the usual dark purple colour of carminate of ammonia. When the free ammonia has evaporated *small* bubbles appear, and the solution takes a brighter red tint.) The solution is left to cool and settle, and by filtering, the bright red deposit (which may be used over again), is separated from the neutral dark fluid, which by the addition of chloral hydrate can be kept for a long time."

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[1] 'Arch. Anat. u. Phys.' (Anat. Abth.), 1882, p. 4.

[2] 'Biol. Centralbl.,' ii (1882), pp. 17—19. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 141.

This preparation has over simple carmine solutions the advantage of keeping well, and some others. "If the solution is mixed with 4—6 times its volume of strong alcohol a scarlet-red precipitate is formed. This is separated by filtration, washed, and dried, or made into a paste with alcohol in which some glycerin and chloral is dissolved. Both the powder and the paste can be kept several months unchanged; they dissolve easily in water, particularly the paste. The solution passes readily through the filter, whilst the ordinary carmine solution can only be filtered with difficulty; it also keeps a long time unchanged, especially with the addition of 1—2 per cent. of chloral, and it has a much more intense colouring power.

"By dissolving the carmine powder in a concentrated solution of neutral picrate of ammonia a combination is obtained which has all the advantages of ordinary picro-carmine without any of its disadvantages."

60. Alum-carmine (*Grenacher's formula*[1]).—An aqueous solution (of 1 to 5 per cent. strength, or any other strength that may be prefered) of common or ammonia alum, is boiled for ten or twenty minutes with ½ to 1 per cent. of powdered carmine. (It is perhaps the safer plan to take the alum solution highly concentrated in the first instance, and after boiling the carmine in it, dilute to the desired strength.) When cool filter.

This stain must be avoided in the case of calcareous structures that it is wished to preserve.

61. Alum-carmine and Acetic Acid.—I learn from M.

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[1] 'Arch. Mik. Anat., xvi (1879), p. 465.

Henneguy that good results are obtained by acidulating alum carmine with acetic acid. (Two or three drops of the acid to a watch-glass of the carmine solution.) This method is in use at the laboratory of Prof. Balbiani.

62. Alum-carmine and Osmic Acid.—In the laboratory of Prof. Bütschli, of Heidelberg, alum-carmine is employed in combination with osmic acid, in the following manner:

To 50 or 60 grammes of water is added alum-carmine until the mixture is of an almost red rose colour; about ten drops of a $\frac{2}{1000}$ solution of osmic acid are then added. (The mixture should have an appreciable smell of osmic acid.) The objects to be stained remain in the mixture for about thirty-six hours, in the dark.

Staining is stated to be more precise than with plain alum-carmine.

I do not know to whom this plan is due.

63. Alum Carmine (*Tangl's formula*[1]).—Powdered carmine boiled in saturated solution of alum for ten minutes and the solution filtered.

64. General Remarks on Picro-carmine.—Picro-carmine-stained preparations should be mounted in balsam, or if in glycerin this should be acidulated with 1 per cent. of acetic or, better, formic acid.

Picro-carmine is a nuclear stain with a tendency to diffuse into formed tissues. If the preparations be washed, after staining, with water, it is a *single* stain, the colour of the carmine alone appearing; if they be washed quickly in alcohol it is a *double* stain, the yellow colouration of the picric acid not being dissolved out by the alcohol as it is by water. Of course the washing with alcohol must not be overdone or the yellow colouration may be entirely removed.

HCl and Picro-carmine (*Neumann's method of using*[2]).—Stain with picro-carmine and wash out with a mixture of glycerin and hydrochloric acid, ½ per cent., or "one or two drops of hydrochloric acid to a few cubic centimetres of glycerin." This is followed by pure glycerin. From half an hour to an hour is generally long enough to enable the hydrochloric acid to have dissolved out the stain from the extra-nuclear elements; but *very deeply* stained preparations may require as much as twenty-four hours. Specimens should be mounted in glycerin rather than in balsam; and the acid must be thoroughly soaked out before mounting. Nuclei are stained carmine red; protoplasm, muscle substance, fibrin of blood, certain colloids and amyloids, the horny substance of epidermis, nails, and hairs, and the ground substance of cartilage, are stained citron-yellow. The intercellular substance of connective tissue, elastic fibres, matrix of bone, mucous tissues, and fat, are not stained at all.

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[1] Pringsheim's 'Jahrb.,' 1880, t. xii. Carnoy, 'La Biologie Cellulaire,' p. 92.

[2] 'Arch. Mik. Anat.,' Bd. xviii (1880), p. 130.

65. Picro-carmine (*Ranvier's formula*[1]).—To a saturated solution of picric acid add carmine (dissolved in ammonia) to saturation. Evaporate down to one fifth the original volume in a drying oven; and separate by filtration the precipitate, poor in carmine, that forms in the liquid when cool. Evaporate the mother liquor to dryness, and you will obtain the picro-carminate in the form of a crystalline powder of the colour of red ochre. It ought to dissolve completely in distilled water; a 1 per cent. solution is best for use.

66. Picro-carmine (*Gage's formula*[2]).—Mr. Gage points out that it is very difficult to decide *when* the mixture of carmine and picric acid solutions become saturated; and that the simple watery solution soon becomes mouldy. Some experiments were made in the anatomical laboratory of the Cornell University, U.S., to determine the formula for a solution that would keep for any length of time.

[1] 'Traité Technique,' p. 100.

[2] 'Am. M. Mic. Journ.,' i (1880), p. 22. 'Journ. Roy. Mic. Soc.,' vol. iii, p. 501.

Take of carmine and of picric acid equal parts by weight.

Dissolve the picric acid in one hundred times its weight of water (using heat if necessary).

Dissolve the carmine in fifty times its weight of strong ammonia.

Mix the two solutions. Use porcelain evaporators and glass funnels.

The best results were obtained when the solutions were made at the ordinary temperature of the laboratory, 17° C., and then evaporated three-fourths at a temperature of 40—45° C. The solution should be allowed to cool, and be filtered through two thicknesses of filter paper. The filtered liquid is then evaporated to dryness at 40° C. or at the ordinary temperature. The residue, dissolved in one hundred times its weight of water, should give a clear solution after filtering.

Make 50 c.c. of such a solution and filter it through two thicknesses of filter paper and a fine cotton filter moistened well and crowded into the neck of the funnel. Filter the solution four or five times through the same filter, and a clear solution will probably be obtained. (*Sic.* I am here copying *verbatim* from the 'Journ. Roy. Mic. Soc.' If it should appear difficult to understand why 50 c.c. of a clear solution should be subjected to all this filtering in view of "probably" obtaining "a clear solution," it may be well to suppose that in directing us to take 50 c.c. "of such a solution" the writer means simply to direct us to take a watery solution, not necessarily clear, and to filter it thus if not clear.) The writer continues:—In case a clear solution cannot be obtained by repeated filtering the whole of the powder may be dissolved in the proportion given above and allowed to stand a few days in a tall narrow vessel. If the finely-suspended particles settle, the top will be clear and may be decanted, but if the fluid remains cloudy a quantity of ammonia equal to that originally used should be added to it, and the evaporation of three-fourths should be repeated with the subsequent filtration and evaporation to dryness.

In case the third evaporation should not give a clear solution it is advisable to begin again with new materials.

When a clear solution is obtained there should be added to every

100 c.c. of the picro-carmine,
25 c.c. of strong glycerin, and
10 c.c. of 95 per cent. alcohol

There will thus be formed a permanent solution that may be kept perfectly clear by filtering once in five or six months.

67. Picro-carmine (*Baber's formula*[1]).

Carmine	1 gramme.
Liq. Ammoniæ	4 c.c.
Water	200 grammes.

Mix and add picric acid 5 grammes. Agitate from time to time during two days, allow residue to settle; decant, and evaporate decanted liquor at the temperature of the air; redissolve the crystals in water (strength 2 per cent.), and filter if necessary.

68. Picro-carmine (*Rutherford's formula*[2]).—Take 100 c.c. of a saturated solution of picric acid. Prepare an ammoniacal solution of carmine by dissolving one gramme in a few c.c. water, with the aid of excess of ammonia and heat. Boil the picric acid solution on a sand bath, and when boiling add the carmine solution. Evaporate to dryness. Dissolve the residue in 100 c.c. water and filter. A clear solution ought to be obtained; if not, add some more ammonia, evaporate, and dissolve as before.

69. Picro-carmine (*Mayer's formula*[3]).—Dissolve of carmine ca 2 grammes in ammonia, and dilute with water to ca 25 c.c.,[4] and leave the solution uncorked for a week or so. To one volume of the solution then add about 4 vols. of a concentrated solution of picric acid in water, or add the solution of picric acid until it ceases to produce a precipitate. Use the resulting mixture for staining.

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[1] 'M. M. J.,' vol. xii, p. 48, and 'Quart. Journ. Mic. Sci.,' 1874, p. 251.

[2] 'Practical Histology,' p. 173.

[3] 'Mitth. d. Zool. Station zu Neapel,' Bd. ii, p. 20.

[4] This appears to be the author's meaning, though his statement is far

The author notes that it is deficient in the penetrating power necessary for thoroughly staining chitinous organisms, but states that it often affords a more *precise* stain than is obtainable by any other means.

70. Picro-carmine (*Pergens' formula*[1]).—Five hundred grammes of cochineal (pulverised) are boiled for two hours and a half in thirty litres of water. Fifty grammes of potassic nitrate are then added, and, after again boiling for a moment, sixty grammes of potassic oxalate; the ebullition is maintained for a quarter of an hour. On cooling, the carmine precipitates; it is washed several times with distilled water. These operations take three or four weeks.

A mixture of 1 vol. caustic ammonia with 4 vols. of water is then poured on the carmine, care being taken that the carmine remain in excess. After two days the mixture is filtered and the filtrate exposed to the air until a precipitate is produced. This is removed by filtration. A saturated aqueous solution of picric acid is then added, the mixture is shaken up and allowed to settle for twenty-four hours. It is then filtered and one gramme of chloral added for each litre of the solution. After eight days the slight precipitate that

from being clear. He writes:—"Es empfiehlt sich, eine gewöhnliche recht starke Carminlösung (etwa 2 g. auf 25 c.cm. Wasser), deren Ammoniak durch wochenlanges Stehen an der Luft verdunstet ist, mit concentrirter wässeriger Picrinsäurelösung so lange zu versetzen, als noch kein Niederschlag entsteht." Whitman directs, "To a mixture of powdered carmine (2 g.) with water (25 c.cm.), while heating over a water-bath, add sufficient ammonia to dissolve the carmine," 'Journ. Roy. Mic. Soc.' (N.S.), ii, p. 876.

[1] Carnoy, 'La Biologie Cellulaire,' p. 92.

has formed is removed by filtration, and the picro-carmine is fit for use.

Carnoy finds this picro-carmine gives better results than any other; it has kept for more than two years without change in his laboratory. He prefers it to Hoyer's.

71. Picro-carmine and Eosin (*Lang's formula*[1]).—Fifty parts 1 per cent. picro-carmine and 50 parts 2 per cent. (aqueous) solution of eosin. Objects are left in the mixture from half a day to four days, according to their permeability. The picrin is then extracted by frequently changed washes of 70 per cent. alcohol; this is followed by 90 per cent. alcohol, which is changed until no more eosin dissolves out.

A double stain, inserted here because the eosin appears to play a peculiar *rôle*, its superior penetrating power enabling it to serve as a vehicle for carrying the picro-carmine through structures which would otherwise be impervious to the latter. This method was found to be the best of all for *Dendrocœla* and other *Platyhelmia*.

72. Picro-carmine (*Weigert's formula*[2]).—Two grammes of carmine are soaked for twenty-four hours (in a spot protected from evaporation, in 4 grammes of ammonia; 200 grammes of concentrated solution of picric acid are then added, and the whole put away for twenty-four hours more. Small quantities of acetic acid are then added "until the first slight precipitate appears even after stirring." The whole is again put away for twenty-four hours more, when it will be found that there has formed a precipitate that can only partially be removed by filtration; ammonia is then added drop by drop at intervals of twenty-four hours, until the solution becomes clear. If the solution stains too yellow, acetic acid is added; if it overstains red, a little ammonia is again added. All badly staining samples of picro-carmine may be improved in the same way by addition of acetic acid.

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[1] 'Zool. Anzeig.,' 1879, p. 45.

[2] 'Virchow's Archiv,' Bd. 84, pp. 275, 315. 'Zool. Jahr.,' 1881, p. 40.

73. Picro-carmine (*Hoyer's formula*[1]).—Made by dissolving in a concentrated solution of neutral picrate of ammonia the carmine powder obtained by precipitating with alcohol the neutral solution of carminate of ammonia (*ante*, formula No. 58). This combination "has all the advantages of ordinary picro-carmine without any of its disadvantages."

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[1] 'Biol. Centralbl.,' ii (1882), pp. 17—19. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 142.

CHAPTER VI.

BORAX-CARMINE (AQUEOUS). ACETO-CARMINE.

74. Borax-carmine (*Woodward's formula*[1]).

Best carmine (No. 40	gr. xv.
Borax	ʒj.
Water	fl. ℥vss.
Alcohol (95 per cent.)	fl. ℥xj.

Mix and filter. "The greater part of the carmine, crystallising in some as yet unstudied combination with the borax, remains on the filter, and the fluid which passes through is comparatively pale and stains but slowly. On the other hand, the crystals which remain on the filter, if dissolved to saturation in distilled water, yield a fluid which stains with great energy," *cf.* 'Monthly Micr. Journ.,' viii, 1872, p. 38. Therefore—Dissolve the crystals in eight ounces of distilled water, and evaporate over a water-bath to four ounces.

Tissues stain in this fluid in a few seconds, but quite uniformly. They are then washed in hydrochloric acid one part, alcohol four parts, until they assume a bright red colouration (which happens in a few seconds). They are then washed in several changes of pure alcohol before mounting.

A nuclear stain, bright red in colour, and permanent both in glycerin and balsam.

75. Borax-carmine (*Heneage Gibbes's formula*[2]).

Carmine	ʒss.

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[1] 'Am. Quart. Mic. Journ.,' i (1879), p. 220. 'Journ. Roy. Mic. Soc.,' ii, p. 613.

[2] Journ. Roy. Mic. Soc.,' iii, 1880, p. 390.

Borax	ʒij.
Aqua	℥iv.

Mix and decant, *not filter.*

Stain in this for a few minutes, wash in—

Hydrochloric acid	1 part.
Absolute alcohol	20 parts.

Until the tissues are of a bright rose colour (this happens in a few seconds).

Then wash in several changes of spirit to remove the acid.

76. Borax-carmine (*Grenacher's formula*[1]).

A 1 to 2 per cent. solution of borax in water is boiled with $\frac{1}{2}$ to $\frac{3}{4}$ per cent. of carmine until a rich dark purple solution is obtained. (If a *clear* solution be not obtained, as may happen in the case of certain sorts of carmine, *filter.*) To the clear solution add *cautiously,* and with continual agitation, drop by drop, *dilute* acetic acid. The colour of the solution becomes more and more carmine-red, and when it has attained to about the redness of the common ammoniacal solutions of carmine, or better, to a slightly redder hue, the addition of acetic acid must be stopped. Leave the solution to settle for twenty-four hours, and decant.

Tissues stain in this in from half to three minutes, but in an incomplete and perfectly diffuse way, the colour being merely precipitated on the surface of the tissues. These must then be rinsed in water and brought into a watch-glassful of (50 to 70 per cent.) alcohol, to which is added one drop of hydrochloric acid. In a few seconds a coloured area will be seen to have formed around the preparations by the re-dissolved carmine, and in a few minutes the preparation may be removed, and will be found to exhibit a precise and perfect staining of the nuclei. A more or less diffuse stain may of course be obtained by shortening the immersion in the HCl. In the case of delicate preparations, a weaker solution of hydrochloric acid should be used, and a correspondingly longer period of immersion.

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[1] 'Arch. Mik. Anat.,' xvi (1879), p. 466.

77. Aceto-carmine (*Schweigger-Seidel's formula*[1]).—To an ammoniacal solution of carmine add acetic acid to neutralisation or even to excess. After separation, by filtration, of the slight precipitate that forms, there is obtained a liquid of a vinous red. Tissues are placed in it for some minutes, then in a mixture of—

Water	200 grammes.
Hydrochloric acid	1 „

Specimens must be mounted either in balsam or in an acid medium; formic acid is to be preferred to acetic acid as the acidulating agent. Ranvier recommends:

Glycerin	100
Formic acid	1

78. Aceto-carmine (*C. S. Minot's formula*[2]).—"Dissolve some finely-powdered carmine in a small quantity of ammonia; add an equal volume of rather strong acetic acid; the exact proportion is not of very great import."

79. Acetic-acid Carmine (*Schneider's formula*[3]).—To boiling acetic acid of 45 per cent. strength, add carmine until no more will dissolve, and filter. (Forty-five per cent. acetic acid is the strength that dissolves the largest proportion of carmine.)

To use the solution dilute it to 1 per cent. strength. The dilute solution may either be used for slow staining, which is the method to be preferred for making glycerin preparations; or, a drop of the concentrated solution may be added to a fresh preparation under the cover-glass. This method only gives certain results with ova of starfishes.

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[1] Ranvier, 'Traité Technique d'Histologie,' p. 99.

[2] 'M. M. J.,' vol. xviii, p. 101.

[3] 'Zool. Anzeig.,' No. 56 (1880), p. 254.

CHAPTER VII.

ALCOHOLIC CARMINE (OXALIC, BORACIC, HYDROCHLORIC, AND SULPHURIC).

80. Oxalic-acid Carmine (*Thiersch's formula*[1]).—Make a solution of 1 part by weight of carmine in 1 of ammonia and 3 of water; to 1 volume of this add 8 volumes of an aqueous solution of oxalic acid of 1 : 22 strength; to this mixture add 12 volumes absolute alcohol, and filter.

The filtrate may be made to assume at will an orange hue, by the addition of oxalic acid, or a violet hue, by the addition of ammonia. Either may be used for staining. If the addition of oxalic acid cause a crystallisation of the acid oxalate of ammonia, this may be dissolved by means of a few drops of distilled water or ammonia; or it may be removed by filtering.

If the concentrated violet fluid be taken for staining, sections are stained in a few moments, uniformly, but cells rather more deeply than other tissue-elements. To stain slowly, the solution must be diluted with alcohol of 70 to 80 per cent.; not with absolute alcohol, which would precipitate the acid oxalate of ammonia. Overstains may be washed out in a few minutes in an alcoholic solution of oxalic acid. The stain is equally applicable to alcohol—and to chromic acid—preparations.

80a. Lilac Borax-carmine Stain (*Thiersch's formula*[2]).—One part of carmine and 4 parts borax are dissolved in 56 parts distilled water. To 1 volume of the solution add 2 volumes absolute alcohol, and filter.

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[1] 'Arch. Mik. Anat.,' i (1865), p. 149.

[2] 'Arch. Mik. Anat.,' i (1865), p. 150.

Stains more slowly than the last formula, oxalic acid carmine (No. 80). Overstains may be washed out with alcoholic solution of borax or oxalic acid. Is particularly applicable to cartilage, and to bone that has been decalcified by chromic acid.

81. Alcoholic Borax-carmine (*Grenacher's formula*[1]).—It is evident that the procedure for employing borax-carmine must be modified if it be wished to stain large pieces of tissue or entire organs *before* cutting sections. In this case, take a *concentrated* solution of carmine in borax solution (2 to 3 per cent. carmine to 4 per cent. borax); dilute it with about an equal volume of 70 per cent. alcohol, allow it to stand some time, and filter. Acetic acid must not be added. Leave the preparations in the stain until they are thoroughly penetrated, and then bring them (*without first washing out*) into alcohol acidulated with 4 to 6 drops of hydrochloric acid to each 100 c.c. of alcohol. They are left in this until they are thoroughly penetrated, and may then be washed or hardened in neutral alcohol.

82. Alcoholic Borax-carmine (*Bourne's formula*[2]).—The directions given by Bourne are somewhat different from those given by Grenacher. A mixture of carmine and borax solution is *allowed to stand for two or three days* and occasionally stirred; the greater part of the carmine will dissolve. To the solution is added an equal bulk of 70 per cent. of alcohol; the mixture is allowed to stand for a week and then is filtered. If on keeping more carmine is deposited it must be refiltered. The tissues may remain in the stain for one, two, or three days, according to size. They should remain in the acidulated alcohol till they acquire "a bright transparent look" (three to six hours).

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[1] 'Arch. Mik. Anat.,' xvi (1879), p. 466, *et seq.*

[2] 'Quart. Journ. Mic. Sci.,' lxxxvii (1882), p. 335.

83. Alcoholic Carmine (*Hoyer's formula*[1]).—Scrapings of carmine ("sonst nicht weiter zu verwendende Carminrückstände") are thrown into a retort with alcohol acidulated with a small percentage of sulphuric acid and kept simmering over a water-bath until the carmine is dissolved. Filter, dilute largely with water, and add solution of sugar of lead so long as the rose-coloured precipitate of sulphide of lead continues to be thrown down. As soon as a *violet* precipitate makes its appearance in the place of the rose-coloured one, filter, and to the filtrate again add solution of sugar of lead so long as the violet precipitate continues to form. This is now collected on a filter, washed, dried, and suspended in a small quantity of strong alcohol. To this is added alcohol, strongly acidulated with sulphuric acid, drop by drop, until the violet precipitate is seen to have lost its colour and the alcohol is become intensely red. Filter, and keep the solution for use. It contains essentially the same colouring matter as the solutions of carmine in acetic, oxalic, or other acids. Two drops of the solution in a watch-glassful of water make a strongly staining liquid. The author gets better results with it than with Rollett's "Carminroth."

84. Alcoholic Carmine (*Grenacher's formula*[2]).—To 50 cubic centimetres of alcohol (60 to 80 per cent.) add 3 to 4 drops of hydrochloric acid and a knife-pointful of powdered carmine. Boil for ten minutes. When cool, filter.

The solution may or may not be now ready for use; this depends on the proportions of acid and carmine used, and these proportions cannot be exactly prescribed on account of the variability of commercial carmine. If the solution is found to give in five or ten minutes a diffuse stain (like a borax-carmine stain, *see* No. 76), more hydrochloric acid must be cautiously added drop by drop, and the solution tested with fresh sections until the desired effect is produced. If after some days (or at once) the solution gets a yellow hue, it is a sign that too much HCl has been used, and the excess must be neutralised by cautious addition of ammonia, which will restore the purple tint of the solution.

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[1] 'Arch. Mik. Anat.,' xiii (1876), p. 650.

[2] 'Arch. Mik. Anat.,' xvi (1879), p. 468.

This is, generally speaking, a nuclear stain. Overstaining and diffusion, should either happen, may be corrected by washing out with alcohol *very slightly acidulated* with HCl. Sections must always be washed in alcohol, not water; and alcohol, not water, must be used for diluting it. Dilute solutions often give results different from those given by concentrated solutions.

85. Alcoholic Carmine (*Mayer's formula*[1]).—A modification of Grenacher's formula, *supra*, No. 84.

Four gr. carmine are dissolved in 100 c.c. of 80 per cent. alcohol with the addition of 30 drops of concentrated pure hydrochloric acid, and heated for about half an hour in the water-bath; the solution is filtered whilst still hot and the superfluous acid is carefully removed by the addition of caustic ammonia, added until the carmine begins to be deposited. This solution stains very rapidly (embryos of lobsters are stained in about a minute) and intensely, though diffusely; the preparations must be washed out with HCl alcohol if a nuclear stain is required. If the preparations be sections mounted on Mayer's albumen fixing medium (No. 276), the precise moment of sufficient washing out may be known by the appearance of the albumen, which will completely give up its carmine to the alcohol or remain at most only faintly coloured.

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[1] 'M. T. Zool. Stat. Neapel,' iv (1883), p. 521. 'Journ. Roy. Mic. Soc.' (N.S.), iv (1884), p. 317.

CHAPTER VIII.

COCHINEAL STAINING SOLUTIONS.

86. Cochineal.—Cochineal appears to have been first recommended as a staining agent by Partsch in 1877. He employed a solution of cochineal in an aqueous solution of alum. A similar formula was again recommended by Czoker in 1880. The formula known as Klein's cochineal fluid (which appears to have been first published in the 'Ann. and Mag. Nat. Hist.,' viii, 1881, p. 232) is identical with that of Czoker. These fluids greatly resemble alum-carmine in their properties, especially in their great selective faculty. It is probably in this point that their chief superiority to alum-carmine is to be found. An important improvement was effected in 1878 by the discovery by Paul Mayer of the properties of the *alcoholic* tincture of cochineal. This preparation puts into our hands a stain that is as precise, as penetrating, and as little hurtful to tissues as Kleinenberg's hæmatoxylin; whilst it has the advantage of being more durable, and above all, of being the easiest to prepare of all staining fluids. Indeed, almost the only point in which it proves inferior to hæmatoxylin and to some carmine fluids, is that it does not in all cases afford a sufficiently powerful stain. (It is not known whether it is permanent in glycerin and similar mounting mediums. I am inclined to doubt its being so; I found some preparations mounted in *acidulated* glycerin became destroyed by fading of the colour in a very short time.)

The alcoholic solution of cochineal possesses a very great selective power. Some tissue elements are stained red, others of different shades of blue. But it is important to observe that these colourations are not constant. They depend upon the presence in the tissues of certain salts or acids, and it is therefore impossible to say beforehand what kind of stain will result. Some workers consider this to be a serious disadvantage. On the whole, this stain may be recommended to the beginner as being probably the safest of all alcoholic staining fluids.

87. Alcoholic Cochineal (*Mayer's formula*[1]).—Cochineal in coarse powder is macerated for several days in alcohol of 70 per cent. For each gramme of the cochineal there is required 8 to 10 c.c. of the alcohol. Stir frequently. Filter, and the resulting clear, deep red solution is fit for staining.

The objects to be stained must previously be imbibed with alcohol of 70 per cent., and alcohol of the same strength must be used for washing out or for diluting the staining solution, as water, or alcohol of a different strength, gives rise to turbidity and precipitation of colouring matter (the fluid holding in solution matters that are only soluble in alcohol of exactly that degree of concentration). The washing out must be repeated with fresh alcohol until the latter takes up no more colour. Warm alcohol acts more rapidly than cold. Overstaining seldom happens; it may be corrected by means of 70 per cent. alcohol, containing $\frac{1}{10}$th per cent. hydrochloric or 1 per cent. acetic acid.

Small objects and thin sections may be stained in a few minutes, larger animals require hours or days. In the latter case large quantities of the solution must be employed. Very thin sections and delicate objects are best stained in a very dilute solution.

A nuclear stain, slightly affecting protoplasm. The colour varies with the reaction of the tissues, and the presence or absence of certain salts. The salts of the metals and alkaline earths that are present in the tissues, and that are soluble in alcohol, give rise to colourations of a bluish tone, so that when these are present the effect is that of a hæmatoxylin stain. In the presence of acids of course the precipitation of these blue combinations in the nuclei and protoplasm cannot occur, and therefore tissues of an acid reaction, as well as those free from the salts in question, stain red. Crustacea with thick chitinous integuments are generally stained red, most other organisms blue. The stain is also often of different colours in different tissue elements of the same preparation. Glands or their secretion often stain grey green. In embryos of *Lumbricus* Kleinenberg found the vessels to stain red, their contents of an intense blue.

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[1] 'Mitth. Zool. Stat. Neap.,' ii (1881), p. 14.

Acids lighten the stain and make it yellowish red. Caustic alkalies turn it to a deep purple.

The best stains are obtained in the case of objects that have been prepared with chromic or picric acid combinations, or with absolute alcohol.[1] The acids must be carefully washed out before staining, or a diffuse stain will result. If it is wished to have the protoplasm strongly stained as well as the nuclei, it is only necessary to wash out incompletely after staining, and to fix the colouring matter by means of strong alcohol. The stain is permanent in oil of cloves and balsam.

The object for which this stain was imagined is twofold. Firstly, to obtain an *alcoholic* stain which enables us to do away with the necessity of treating with an *aqueous* fluid objects that have been preserved in alcohol and that are intended for mounting in balsam, aqueous fluids being often most deleterious to delicate structures. Secondly, to obtain a fluid whose high penetrating power allows it to be employed in the case of organisms, such as Arthropoda, whose chitinous investments are but very slightly permeable by aqueous solutions of carmine.

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[1] Osmic acid preparations stain very weakly unless they have been previously *bleached* (No. 476).

The stain is not so powerful as ammonia carmine or Kleinenberg's hæmatoxylin or Grenacher's alcoholic carmine. In general, however, Mayer prefers it to all other stains, and only uses Kleinenberg's hæmatoxylin in special cases.

Alcohol of 70 per cent. is not the only strength by means of which the colouring matters of cochineal may be extracted. But extracts obtained by means of 90 per cent. or absolute alcohol, are very weak in colour, and are further useless because they yield a diffuse stain. The weaker the alcohol the stronger the extract of colouring matter; and extracts made by means of 50 per cent. or 60 per cent. alcohol would be preferable to that obtained by means of the 70 per cent. alcohol, were it not that they are naturally more deficient in penetrating power.

88. Alum Cochineal (*Partsch's formula*[1]).—Powdered cochineal is boiled for some time in a 5 per cent. solution of alum, the decoction filtered, and a little salicylic acid added to preserve it from mould.

89. Alum Cochineal (*Czoker's formula*[2]).—Seven gr. cochineal and 7 gr. calcined alum are rubbed up together into powder in a mortar, add 700 gr. distilled water, and boil down to 400 gr. When cool, add sufficient carbolic acid to be perceptible by the smell, and filter several times. The violet solution is ready for use, and will keep for six months, after which time it must be filtered again, and a fresh trace of carbolic acid added.

This stain possesses considerable elective faculty, and is stated to stain, in a longer or shorter time, all kinds of tissue, *no matter in what way they may have been hardened.* Nuclei are stained hæmatoxylin-colour, and other elements different tones of red, so that the effect is that of a double stain with hæmatoxylin and carmine. Alcohol objects require three to five minutes, chromic objects three to five hours. The author points out that commercial *carmine* is now of a different quality to that which was obtainable some years ago; and that from the sort which is now usually sold it is not possible to obtain good staining solutions.

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[1] 'Arch. Mik. Anat.,' xiv (1877), p. 180.

[2] 'Arch. Mik. Anat.,' xviii (1880), p. 413.

CHAPTER IX.

HÆMATOXYLIN STAINS.

90. Hæmatoxylin.—For a statement of the chemistry of hæmatoxylin, so far as it bears on the practice of staining, the reader is referred to the exposition of Dr. Cook, *post*, No. 93.

The advantages of hæmatoxylin solutions are chiefly these: that, when properly employed, they afford a most precise and powerful stain; that their staining is not impeded by the previous emyloyment of chromic acid or other similar hardening agents; and (as regards the alcoholic solutions) that they have a very high degree of penetration and are not hurtful to tissues. The disadvantages are: that it is difficult or impossible to get the solutions to keep; and that as preparing them is by no means a simple operation, the necessity of frequently preparing fresh fluid causes serious waste of time. To this must be added that the stains are liable to fade, unless special precautions be taken to free the tissues from all acid before staining and to dehydrate thoroughly before mounting (*see* below Nos. 92 and 93[1]).

The most important of these fluids is the alcoholic hæmatoxylin of Kleinenberg. Kleinenberg has given two formulæ.

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[1] A. C. Cole says, "So far as can be judged by our present data a preparation stained with logwood and mounted in balsam is unchangeable," but "logwood slides mounted in dammar varnish" would be found after about ten years "to be little better than fine grey dust!" Cole's 'Methods of Micr. Research,' pt. vii (1884), p. xli; 'Journ. Roy. Mic. Soc.' (N.S.), 1884, p. 310.

I quote the first from Foster and Balfour's 'Elements of Embryology' (French edition), not knowing where it was first published.

91. Alcoholic Hæmatoxylin (*Kleinenberg's first formula*[1]).—*a.* Make a saturated solution of crystallised chloride of calcium in 70 per cent. alcohol, and add alum to saturation.

b. Make a saturated solution of alum in 70 per cent. alcohol and add 1 volume of the former (*a*) to eight of the latter (*b*).

c. To the mixture thus obtained add *a few* drops of a *barely* alkaline saturated solution of hæmatoxylin. (This should be a saturated solution in absolute alcohol.)

This method was afterwards simplified by the omission of the alum from (*b*), and now runs as follows:

92. Alcoholic Hæmatoxylin (*Kleinenberg's second formula*[2]).—"Prepare a saturated solution of calcium chloride in 70 per cent. alcohol with the addition of a little alum; after having filtered, mix a volume of this with from 6 to 8 volumes of 70 per cent. alcohol. At the time of using the liquid pour into it as many drops of a concentrated solution of hæmatoxylin in absolute alcohol as are sufficient to give the required colour to the preparation of greater or less intensity, according to desire."

At Naples, according to Mayer ('Mitth.,' ii, 1881, p. 13), the solution of calcium chloride is used *saturated* with alum.

Mayer further states that the object of the chloride of calcium is explained by Kleinenberg to be the setting up of diffusion currents between the alcohol in the tissues and the external staining medium, so as to facilitate the penetration of the latter.

Mayer points out that by the reaction of alum and calcium chloride there is formed a precipitate of sulphate of lime, and that it will therefore probably be found better to employ chloride of aluminium in the place of alum.

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[1] 'Eléments d'Embryologie,' de Foster et Balfour, 1877, p. 296.

[2] 'Quart. Journ. Mic. Sci.,' lxxiv (1879), p. 208.

A powerful, nuclear stain. The stain is permanent, *provided (Mayer) that the tissues have been perfectly freed from acid before staining.*[1] The solution itself is not permanent. A successfully prepared fresh solution should be of a violet colour with a decided touch of blue, and should not be at all reddish. If it becomes reddish after standing for some time that is generally because it has become somewhat acid, and the fault may be corrected by holding over the mouth of the bottle containing the solution the stopper of an ammonia bottle; then on shaking up with the solution the small quantity of vapour of ammonia given off from the stopper, the proper colour is generally regained.

Small objects are best stained slowly with a very dilute solution. If it be required to dilute a solution already prepared for staining this should not be done with alcohol, which may easily cause precipitates to form on the tissues, but with the above-described solution of alum in calcium chloride solution. Overstains should be washed out with acidulated alcohol. Either oxalic or ($\frac{1}{2}$ per cent.) hydrochloric acid may be used, and the specimens allowed to remain in them until they begin to acquire a reddish hue. The acid is then removed by pure alcohol which restores the pure blue of the stain.

For large or impermeable objects immersion for days in a very strong solution may be necessary for staining. Osmium and chromic acid objects stain sufficiently.

93. Cupric-sulphate Hæmatoxylin (*Cook's formula*[2]).—

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[1] Heneage Gibbes states ('Journ. Roy. Mic. Soc.,' iii, p. 390) that if preparations which have been stained with picro-carmine be placed "in plain water acidulated with a few drops of acetic or picric acid before staining with logwood, they take the second stain better and *do not fade afterwards.*" It seems that the theory of hæmatoxylin staining is still in a somewhat chaotic state.

[2] 'Journ of Anat. and Physiol.,' xiv (1879), p. 140.

"The colouring material of logwood consists of two substances, hæmatoxylin and hæmatein, differing from one another by two equivalents of hydrogen. Hæmatoxylin is soluble in alum solution, whilst hæmatein is hardly at all so. The latter is of no use to the histologist for colouring animal tissues. Hæmatoxylin forms compounds with various metallic oxides, which are soluble in alum solution also, and if a tissue be stained with hæmatoxylin, or with hæmatoxylin and a metallic oxide and immersed in an aqueous solution of alum, the colour will be all discharged from the tissue and taken up by the solution, and the solution of alum will thus take up fresh quantities of hæmatoxylin compound until it reaches a point of saturation beyond which it will take up no more from the tissues, but will, if over-saturated, give up the colouring matter freely to immersed animal material. Such a solution of hæmatoxylin, alum, and metallic oxide has a clear purple colour, becoming red on addition of acids. If alkaline earths, alumina, or hydrated earthy phosphates be suspended in it, they will absorb the colour and the solution becomes purple. If the solution be treated with a very small percentage of a chromate, the purple will be replaced by a yellowish-brown colour; or if a tissue which has been stained with alum logwood solution be immersed in an exceedingly dilute bichromate solution, the purple will sooner or later be replaced by the yellow tint. If a section of any abnormal caseous concretion or abnormal growth be immersed in a neutral solution of alum logwood, it will become of a more bluish purple than ordinary tissue, evidently from the presence in it of more than an ordinary amount of alkaline earthy matter or phosphate.

" When the above facts are taken into consideration it will appear unreasonable to expect tissues hardened in chromic solutions of any kind to colour as readily with an ordinary logwood solution as they would do if immersed in the fresh state, notwithstanding the assertion of any experimenters to the contrary. Sections of chromic-acid hardened tissues are exceptionally difficult to free from chromic compounds, most probably because part of the chromic acid is in chemical combination and insoluble, and when freed from the hardening material the tissues will not be left in the natural neutral state, and thus less readily will the nuclei take up the colour. But it has been found that hardened tissues, if cut into sections and well washed, may be as readily stained with logwood as fresh tissues if the solution be slightly modified.

" Take—	Logwood extract	6 parts
	Alum	6 "
	Sulphate of Copper	1 "
	Water	40 "

" All ingredients must be free from iron.

" Grind the alum, logwood extract, and sulphate of copper in a mortar, and when powdered add sufficient water to form a thin paste, leave for one or two days with occasional stirring, and then filter. The hæmatein contained in the logwood extract will be retained by the filter with the dirt, and the solution consists of hæmatoxylin, alum, and sulphate of copper, to which a crystal of thymol may be added to preserve it from mould.

" Fresh or alcohol-hardened tissues may be stained with this after sufficient dilution; but for chromic-hardened tissues dilute 8 drops with 120 drops of water, and add one drop of $\frac{1}{10}$ per cent. solution of bicarbonate of potash just prior to use. Wash the stained solutions (*sic!*) in water as usual. N.B.—A larger proportion of bichromate solution will produce an ugly yellow; and if the mixed solution be kept many hours some decomposition will go on.

" Tissues stained in logwood may be mounted in glycerin or Farrant's solution, or in dammar. In the two former they keep unchanged for any length of time, in the latter they are apt to fade unless care be taken, in preparing them for dammar, that the sections be thoroughly freed from water by absolute alcohol before being brought into contact with oil of cloves. If any moisture be left fading will soon commence and the preparation be spoiled."

94. Alum Hæmatoxylin (*Bochmer's formula*[1]).—Make (A) a solution of pure hæmatoxylin (℈j) in absolute alcohol (℥ss) and (B) a solution of alumen deparatum (gr. ij) in water (℥j). Add 2 to 3 drops of A to a watch-glassful of B. The sections may remain in this for half a day to a day. They are then treated as follows: absolute alcohol, solution of tartaric acid in alcohol, absolute alcohol again, then benzine or turpentine, and, finally, mounted in castor-oil.

Other methods of treatment may be employed, provided that hydrated acids and resinous mounting media be avoided, as both are detrimental to the colour of the stain. With these precautions, however, the colour is permanent in glycerin.[2] Alcoholic extract of logwood only imperfectly surrogates the alcoholic solution of hæmatoxylin.

To stain objects that have been treated with chromic acid or its salts or with copper sulphate, &c., the solution A may be diluted with pure water instead of the alum solution; but the stain is more diffuse, and less strongly held by the nuclei.

95. Alum Hæmatoxylin (Aqueous) (*Duval's formula*[3]).—"One grain of concentrated solution of hæmatoxylin in alcohol is added to about 800 grammes of water containing a little alum. There is thus obtained a solution that stains nuclei in a few minutes."

96. Hæmatoxylin Staining Solution (Aqueous) (*Mitchell's formula*[4]).

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[1] 'Arch. Mik. Anat.,' iv (1868), p. 345.

[2] Flemming finds the stain not permanent for more than a year either in resinous media or in glycerin, that is to say, the stain loses in sharpness. 'Zellsubstanz, &c.,' p. 384.

[3] 'Précis Technique,' p. 220.

[4] 'Proc. Acad. Nat. Sci. Philad.,' 1883. 'Journ. Roy. Mic. Soc.' (N.S.), iv, 1884, p. 311.

℞	Finely-ground logwood	℥ij.
	Sulph. alumin. and potash	℥ix.
	Glycerin	f℥iv.
	Water	a sufficient quantity.

"Moisten the ground logwood with sufficient cold water to slightly dampen it, place it in a funnel or percolator, packing it loosely, and then percolate sufficient water through the drug until the liquid coming from the percolator is but slightly coloured. Allow the drug to drain thoroughly, and then remove it from the percolator and spread out on a paper or board to dry. Dissolve the alum in eight fluid ounces of water, moisten the dry drug with a sufficient quantity of the fluid and again pack in the percolator, this time rather tightly, and pour on the remainder of the alum solution. As soon as the liquid percolates through and commences to drop from the end of the percolator, close the aperture with a tightly-fitting cork and allow the drug to macerate for forty-eight hours. Remove the cork at the expiration of that time, allow the liquid to drain off, and then pour sufficient water upon the drug to percolate through twelve fluid ounces altogether. Mix this with the glycerin, filter, and place in a close-stopped (*sic*) bottle."

The fluid may be used undiluted, "but the best results are obtained by placing the tissues in a weak solution (ten drops to two fluid drachms) with warm distilled water for about twelve hours."

The chief object of this process is to obtain a *stable* fluid. In the usual fluids prepared from extract of logwood, "the partially oxidised tannin in the liquid gradually absorbs more oxygen from the air, and changes to other complex organic compounds; the colouring matter is also affected by the decomposition and gradually becomes converted into other substances, and the liquid finally becomes of a dirty muddy colour and is half filled with a lumpy sediment. . . The idea therefore occurred to the author that if the tannin could be removed and the lake of logwood isolated . . a staining fluid could be prepared which would be both permanent and satisfactory. . . In this process nearly all the tannin is removed by percolating the drug with cold water, a menstruum in which the colouring principle is not very soluble, and the subsequent maceration and percolation with the alum solution removes the logwood lake in a state of comparative purity. The glycerin is added simply for its preservative qualities, which may be increased by the addition of a few drachms of alcohol to the solution."

The fluid thus prepared is of a deep purplish red colour. It will keep its colour for a length of time, and deposits no sediment. The stain given by it is of a delicate violet, and Mitchell states is "unrivalled in the delicacy and clearness of differentiation of its colouring."

97. Alum Hæmatoxylin (Aqueous) (*Grenacher's formula*[1]). —To 150 c.c. of a concentrated solution of ammonia alum, add 4 c.c. of a concentrated solution of crystallised hæmatoxylin in absolute alcohol. Leave the mixture exposed to the light for several days, and then add 25 c.c. of methyl alcohol and 25 c.c. of glycerin.

This fluid is chiefly employed for staining sections. It is a fine nuclear stain. Sections should only remain for a very short time in the fluid.

It is best to leave the solution standing until a deposit forms before using.

Flemming finds the stain loses in sharpness after the lapse of a year, both in preparations mounted in resinous media and in glycerin preparations (l. c., p. 384).

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[1] Flemming, 'Zellsubstanz, &c.,' p. 383.

CHAPTER X.

PURPURIN, INDIGO-CARMINE, SAFFRON, ORCHELLA, INK.

98. Purpurin.[1]—Purpurin was first introduced to histological practice by Ranvier, who found in it an important aid to the study of cartilage. It is a colouring matter extracted from garance, and is obtained in the form of a pulverulent body in a state of starch-like agglomeration. It is soluble in a boiling aqueous solution of alum, from which it normally precipitates on cooling, but may be prevented from so doing by the addition of a certain proportion of alcohol. The employment of an alum solution as vehicle for the colouring matter has the advantage, at least so far as cartilage is concerned, of fixing the cellular elements at the same time that they are stained. (Ranvier found that alum in a solution of 5—1000 was the best of all fixing agents for cartilage-cells, 'Traité,' p. 279.)

99. Alum Purpurin Staining Solution (*Ranvier's formula*[2]).—200 grammes of water and 1 of alum are boiled in a porcelain capsule; purpurin rubbed up in water is added, and the boiling continued. The purpurin being dissolved to saturation (this is ensured by taking care to have an undissolved excess in the capsule), the solution is filtered hot into a flask containing 60 c.c. of alcohol (36° Cartier).

There is thus obtained a solution of an orange-rose colour, presenting a marked degree of fluorescence.

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[1] 'Archives de Physiologie,' 1874, p. 761. 'Traité Technique,' p. 280.

[2] 'Traité Technique,' p. 280.

(As regards the quantity of alcohol to be taken, Duval writes that it should always be one-fourth in volume of the total mixture, v. 'Précis de Technique Histologique,' p. 221).

The solution does not keep well for more than a few weeks.

Sections of fresh cartilage are to be placed in a small quantity (only a few cubic centimetres) of the solution, and after remaining there twenty-four to forty-eight hours, are washed in water and mounted in glycerin. The stain is nuclear, the matrix remaining almost colourless. Duval (l. c.) states that this stain has a special selective action on sections of central nervous system (especially spinal cord) obtained from tissues hardened in bichromate of ammonia (2—1000), and mounted, after staining for forty-eight hours, in Canada balsam. The nerve-cells and processes, axis cylinders and fibres of connective tissue are unstained; but the nuclei of connective tissue and of the capillaries are stained red.

100. Glycerin Alum Purpurin (*Grenacher's formula*[1]).—In 50 cubic centimetres of glycerin (pure or diluted with very little water) dissolve from 1 to 3 per cent. powdered alum; add a knife-pointful of purpurin, and boil. (Alcohol must *not* be added.) Let the orange-coloured fluorescent solution stand for two or three days, and then filter.

A nuclear stain; ten to thirty minutes generally suffice to produce good staining. The solution is stable, which Grenacher finds that Ranvier's solution (No. 99) is not, the latter precipitating after a few days.

101. Indigo.—Indigo is employed in histology in the form of solutions of so-called indigo-carmine, or sulphindigotate of soda or potash. The simple aqueous solution gives a diffuse stain, and is therefore not capable of being usefully employed *alone.* It is, however, of great use when employed to bring about a *double* stain in conjunction with carmine. Though it has no selective preference for nuclei or protoplasm, it possesses to a high degree the property of imparting different hues and intensity of stain to different tissues; and the nuclei being brought out by carmine, preparations are obtained of a diagrammatic clearness that is not afforded by carmine alone.

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[1] 'Arch. Mik. Anat.,' xvi (1879), p. 470.

Combined with oxalic acid (Thiersch), indigo-carmine can be made to afford a nuclear stain.

Indigo-carmine is found in commerce in the form of a blue paste, soluble in water. It not unfrequently contains impurities, and it is, therefore, better to prepare it oneself. The following statements concerning commercial indigo-carmine are taken from Heidenhain's paper on the kidney, in 'Arch. Mik. Anat.,' x (1874). The article known in commerce as sulphindigotate of soda is a variable mixture of different substances, of which the chief are generally these three: the soda (or potash) compounds of sulphindigotic acid (sulphocoruleïnic acid), of hyposulphindigotic acid (hyposulphocoruleïnic acid), and of sulphophœnicinic acid. The sulphindigotate of soda is freely soluble in water, slightly soluble in weak alcohol, and as good as insoluble in absolute alcohol. It is precipitated from its solutions by concentrated solutions of salts.

The hyposulphindigotate of soda is equally soluble in water and in absolute alcohol, and is not precipitated by neutral salts. This combination is *frequently* present as an impurity in the sulphindigotate of commerce, which then appears partly soluble in absolute alcohol.

The sulphophœnicate of soda is much less soluble in water than the sulphindigotate, is easily soluble in alcohol, and is easily precipitated by small quantities of salts.

The following method of preparing the pure sulphindigotate is recommended (after Crum and Berzelius) by Maschke:

102. Indigo-carmine.[1]—One part of best powdered indigo is gradually shaken into a large flask containing 7 to 8 parts of pure sulphuric acid of 1,840 sp. gr. As soon as the mass has ceased to rise, the flask is closed and allowed to remain for three days with frequent agitation. The solution is then diluted with 30 to 40 vols. water, allowed to settle, filtered, and to the clear liquid so many parts by weight of crystallised carbonate of soda added, as were taken of sulphuric acid in the first instance. (The soda must be added gradually and in a large vessel on account of the violent foaming. It would be therefore better to use acetate of soda, chloride of sodium, or sulphate of soda instead; the formation and separation of the sulphindigotate of soda being obtainable by almost all soda salts that do not act destructively on the sulphindigotic acid.) Now filter, allow the substance retained on the filter to drain, and throw it into a considerable quantity of 5 per cent. solution of acetate of soda, stirring well. Repeat this operation once or twice.

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[1] 'Arch. Mik. Anat.,' x (1874), p. 32.

The sulphindigotate of soda is now almost freed from the hyposulphindigotate. It should be allowed to drain, the damp mass dried over a water-bath, pulverised, and treated repeatedly with absolute alcohol, by which means the last traces of the hyposulphindigotate, the greater part of the acetate of soda, and the remaining indigo-red, if any, are got rid of.

Another mode of preparation of sulphindigotate of soda is the following, as reported by Seiler:

103. Sulphindigotate of Soda (*Bullock's process*[1]).—"Best Bengal indigo is digested with Nordhausen's sulphuric acid. The excess of acid is then removed by washing, the colouring matter precipitated with chloride of sodium, and left standing for several days. The precipitate is then separated from the mother liquor by filtering through flannel, and the excess of chloride of sodium washed out by pouring cold water through the filter until the colouring matter begins to dissolve. The washing is then stopped, and the precipitate dissolved in warm distilled water to saturation, which makes a solution of a deep greenish-blue colour."

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[1] 'Am. Quart. Mic. Journ.,' i (1879), p. 220. 'Journ. Roy. Mic. Soc.,' ii (1879), p. 614.

104. Carmine and Indigo-carmine Stain (*Seiler's method*[1]).—Specimens are stained with Woodward's borax-carmine, and washed out with HCl, as directed *ante* No. 74. The acid is removed by thoroughly washing in alcohol, and the specimens are then brought into a mixture of two drops of the sulphindigotate of soda solution (*supra* 103) in one ounce of 95 per cent. alcohol. The mixture should be filtered before using. After six to eight hours in the stain the sections are dehydrated with alcohol and mounted.

"The effect of this mode of staining is to leave the nuclei bright red, while the formed material of the cell is slightly tinged with blue. Connective-tissue fibres become stained with a deep blue colour, while the blood-vessels are purplish and mapped out with surprising distinctness. Epithelium and hair take this stain in a very curious manner, inasmuch as the cells of different ages take different colours, ranging from a brilliant emerald green to purple violet and olive green. . ."

105. Oxalic Acid Indigo-carmine (*Thiersch's formula*[2]).—Make a saturated solution of commercial indigo-carmine (sulphindigotate of potash) in solution of oxalic acid of 1 : 22 to 30. Dilute, if desired, with alcohol. Concentrated, the solution stains very intensely in a few seconds; nuclei and protoplasm being stained more deeply than other elements. Overstains may be washed out with alcoholic solution of oxalic acid.

106. Tincture of saffron (*H. Blanc's formula*[3]).—Dissolve 5 grammes of saffron in 15 c.c. of absolute alcohol; allow the solution to settle for a few days, filter, and dilute with one half of water,

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[1] 'Am. Quart. Mic. Journ.,' i (1879), p. 220. 'Journ. Roy. Mic. Soc.,' ii (1879), p. 613.

[2] 'Arch. Mik. Anat.,' i (1865), p. 150.

[3] 'Zool. Anzeig.,' 129 (1883), p. 23.

For *Protozoa*.—The organisms having been fixed with solution of Kleinenberg, and washed out with successive alcohols up to absolute alcohol, are stained in the saffron solution; they are then washed out with 80 per cent. alcohol, the washing out being continued until the colouring matter is sufficiently removed from the protoplasm; as soon as this is seen to be the case absolute alcohol followed by oil of cloves is substituted for the 80 per cent. alcohol, and the preparations when cleared are mounted in balsam. A nuclear or protoplasmic stain, which Blanc prefers to picro-carmine because its action is more rapid, and can be controlled more precisely by means of washing out according as it is desired to stain the protoplasm or the nuclei alone.

107. Orchella (*Wedl's formula*[1]).—French orchella extract, from which the excess of ammonia has been removed by gentle warming in a sand-bath, is poured into a mixture of absolute alcohol 20 c.c., acetic acid (concentrated, of 1·070 sp. gr.) 5 c.c., and water 40 c.c., until a saturated dark-red stain is obtained, which must then be filtered once or twice. Sections are washed with water, drained, and treated with the stain. Mount in levulose. A protoplasmic stain, nuclei remaining colourless. Connective-tissue cells stain deeply, the intercellular substance less deeply. Epithelia, if horny or calcareous, are not stained. The basic substance of bone and teeth take the stain, and so do ganglion-cells and their processes.

108. Ink Process (*Paul's method*[2]).—Stephens' "blue-black" ink is the ink usually employed. It must be quite fresh. Sections of spinal cord (chromic acid preparations) are stained for a few minutes in a solution of 1 part of the ink to 5 or 10 of water; or for a few hours in a solution of 1 : 30 or 1 : 50. Mount in balsam.

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[1] 'Arch. f. path. Anat.,' lxxiv, p. 143. 'Journ. Roy. Mic. Soc.,' ii, 1879. For an account of this substance *vide* Cooley's 'Cyclopædia,' *sub voce* 'Archil.'

[2] Marsh's 'Section-cutting,' 2nd ed. (1882), p. 148.

For spinal cord.

108a. Bilberry Juice (*Lavdowsky's method*[1]).—Fresh berries of *Vaccinium myrtillus* (the common bilberry or whortleberry) should be well washed in water and the juice expressed and mixed with two vols. of distilled water to which "some" 90 per cent. alcohol has been added. It is then heated for a short time and filtered warm. For use, a small quantity should be diluted with two or three vols. of distilled water.

The stain gives a red colour with fresh neutral objects, or lilac when the acid of the fluid is neutralised by an alkali or neutral salt. The latter is the more durable. The stain may be followed by eosin to procure a double stain.

It stains well the nuclei of all cells, and shows karyokinetic figures "very plainly."

The reporter of the 'Journ. Roy. Mic. Soc.' criticises the publication of this stain (which he considers to be probably useless) as an instance of "the modern fashion of recommending every conceivable substance which by any chance will furnish a stain." I consider the criticism unfortunate. A stain that is capable of showing karyokinetic figures plainly in fresh objects is certainly not useless, and it appears improbable that so accomplished an histologist as Lavdowsky should take trouble to recommend a useless process.

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[1] 'Arch. Mik. Anat.,' xxiii (1884), pp. 506-8. 'Journ. Roy. Mic. Soc.' (N.S.), iv (1884), p. 652.

CHAPTER XI.

GOLD CHLORIDE.

109. Gold Chloride (*Cohnheim's method*[1]).—The well-known "gold method" was invented by Cohnheim. He practised it as follows: Fresh pieces of cornea (or other tissue to be operated on) are put into solution of chloride of gold of 0·5 per cent. strength until they are thoroughly yellow, and then exposed to the light in water acidulated with acetic acid until the gold is thoroughly reduced, which happens in the course of a few days at latest. They are then mounted in acidulated glycerin.

The method in this, its primitive form, often gave splendid results, but was very uncertain, giving sometimes a nuclear or protoplasmic stain, sometimes an extra-cellular impregnation similar to that of nitrate of silver. And the preparations thus obtained are not permanent, seldom keeping for more than a few months.

Of late years very important improvements in the gold process have been worked out. In order to facilitate the penetration of the gold and its subsequent reduction in the tissues, the tissues are made to swell up by treatment with formic acid before being brought into the gold bath, and formic acid is employed to assist the reduction after impregnation. This is the principle of the process known as the "method of Löwit." Quite recently this method has been, as it seems to the author, improved in important respects by the employment of fixing agents before the formic acid or in conjunction with it, or other acid used in place of it, and by employing a very dilute solution of the gold salt.

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[1] 'Virchow's Arch.,' Bd. xxxviii, pp. 346—349. 'Stricker's Handb.,' p. 1100.

Thus employed, gold chloride is a most important aid to the study of the peripheral parts of nervous systems, which are by this means stained of a beautiful violet, surrounding tissues remaining colourless, so that the differentiation of the nerve-fibres is as perfect as possible. But even when thus employed with all possible precautions I consider gold chloride to be uncertain in its action, and that the results obtained by means of it need to be controlled by the employment of other methods. As an instance in point I may quote one of the results of the researches of Viallanes into the peripheral nervous system of the larvæ of *Diptera*. That accomplished histologist employed the excellent modification of the gold method described below. He was led by it to describe as peripheral ganglia, of a form that he calls melon-ribbed (*à côtes de melon*), ganglia of larvæ of *Tipula*, *Musca*, and *Eristalis*, that from the position he assigns to them can be none other than the chordotonal ganglia, for that position is just that occupied by the chordotonal organs in these species, and no other composite ganglia exist in that position. He even succeeded in obtaining a sight of the scolopal bodies, and describes them (*Musca*, *Eristalis*) as "small fusiform bodies stained of a deep violet, and having in their centre a round spot completely colourless." The reader needs only to compare this description with the complex structure found in these bodies by various German writers, and recently expounded at large by Graber ('Arch. Mik. Anat.,' Bd. xx), and by myself (*ibid.*, Bd. xxiii), and to remember that all the details of these elements were worked out by simply placing the living animal in a compressorium, or, better still, he needs but to devote half an afternoon to the study of a small larva of *Eristalis* in a suitable compressorium, in order to be convinced that the gold method is not infallible. (I tried it myself in my researches on these chordotonal organs, accord-to Viallanes' plan; it gave quite worthless results. Osmic acid and tincture of cochineal were the only stains that gave me fair preparations. (See a paper by the author on this subject in 'Recueil Zoologique Suisse,' t. i, 1884.)

Gold chloride may be followed by a carmine stain for the demonstration of nuclei, if the nuclei are not already impregnated by the gold. I believe the preparations are permanent if made carefully according to a good formula, such as that of Viallanes. They may be mounted in acidulated glycerin (with 1 per cent. formic acid) or in balsam.

One of the chief defects of gold staining is the *over-colouration* which frequently renders preparations useless after a short time. Ranvier states that this can be avoided by putting the preparations for a few days into alcohol, which possesses the property of stopping the reduction of the gold.

Instead of the simple salt it is generally found better to employ the double chloride of gold and potassium or sodium, the details of the process being in either case the same. The reasons for this are explained by Hoyer, *infra*, No. 111.

110. Gold Chloride (*Chrschtschonowic's method*[1]).—(For nerves of buccal mucosa.) Elin describes this method, l. c. Fresh pieces of mucosa are treated as follows: Gold chloride of ½ per cent. thirty minutes; water six to twenty-four hours; nearly saturated solution of tartaric acid, kept at a temperature of 40°—50° C., until complete reduction; water a few minutes; alcohol until hardened fit for section cutting.

111. Double Chloride of Gold and Potassium (*Hoyer's method*[2]).—(For corneal nerves.) The double chloride of gold and potassium has the following advantages over the simple gold chloride. It is more easy to be obtained of unvarying composition, it is more perfectly neutral, and its solutions are more perfectly stable. It is used in solutions of the same strength as chloride of gold, viz. 0·5 per cent. Corneæ must be very thoroughly imbibed with the solution. Small corneæ (rabbit, guinea-pig) require half to one hour, human corneæ two to five hours (in an acidulated solution). It is better to err on the side of too-prolonged immersion, rather than the contrary. In order to demonstrate the intra-epithelial ramifications of nerves, the gold is partially reduced by exposure for sixteen to twenty-four hours in (1 or 2 ounces of) distilled water, and there is added to the water one or two drops of a pyrogallic-acid developing solution, such as is used in photography (*vide* Gerlach, 'Die Photographie als Hülfsmittel der mikroskopischen Forschung,' Leipzig, 1863). Or instead of treating them with the developing solution, the corneæ may be removed to a warm, concentrated solution of tartaric acid and remain there at the temperature of an incubating stove until the gold is fully reduced.

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[1] 'Arch. Mik. Anat.,' vii (1872), p. 383.

[2] 'Arch. Mik. Anat.,' ix (1873), p. 222.

112. Chloride of Gold (*Löwit's method*[1]).—I take the following directions as to this method from Fischer's paper on the tactile corpuscles of Meissner. Löwit's method was first published by him in the 'Wien. Sitzgsber.,' lxxi Bd. iii Abth., 1875, p. 1.

Small pieces of *fresh* skin are put into dilute formic acid (one volume of water to one of the acid of 1·12 sp. gr.), and remain there until the epidermis peels off. They then are put for fifteen minutes into gold-chloride solution (1½ per cent. to 1 per cent.), then for twenty-four hours into dilute formic acid (1 part of the acid to 1—3 of water), and then for twenty-four hours into undiluted formic acid. (Both of these stages are gone through in the dark.) Thin sections are then made and mounted in dammar or glycerin. Successful preparations show the nerves alone stained, but it is not possible always to control the results. After impregnation, tissues may be stained for demonstration of nuclei with carmine, hæmatoxylin, or anilin. The best results were obtained with anilin applied in the manner described by Hermann (in 'Tageblatt der 48 Versammlung deutscher Naturforscher in Graz,' 1875, No. 4, p. 105, *vide post*, 'Anilin Stains'). This method has the advantage of procuring a purely nuclear stain.

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[1] 'Arch. Mik. Anat.,' xii (1875), p. 366.

113. Gold Chloride (*Ranvier's method*[1]).—The method of Löwit has been modified by many workers by omitting the final treatment with undiluted formic acid, and also in some other details. Ranvier proceeds as follows. Reflecting that the action of the one-third formic acid in which Löwit placed his tissues must be hurtful to the finer ramifications of the nerves, he combines the formic acid with a fixing agent designed to antagonise its macerating action, and takes for this purpose the chloride of gold itself. The tissues are placed in a mixture of chloride of gold and formic acid (4 parts of 1 per cent. gold chloride to 1 part of formic acid) which has been boiled and allowed to cool.[2] They remain in this until thoroughly impregnated (muscle twenty minutes, epidermis two to four hours); the reduction of the gold is effected either by the action of daylight in acidulated water, or in the dark in dilute formic acid (1 part of the acid to 4 parts of water).

The object of boiling the mixture of gold chloride and formic acid is this, that "by boiling in the presence of the acid the gold acquires a great tendency to reduction, and for this reason its selective action on nervous tissues is enhanced."

114. Gold Chloride and Lemon-juice (*Ranvier's method*[3]).—Instead of combining the formic acid with gold chloride in order to mitigate its action, recourse may be had to a less injurious acid than formic acid. Ranvier finds that of all acids lemon-juice is the least hurtful to nerve-endings. He therefore soaks pieces of tissue in fresh lemon-juice filtered through flannel, until they become transparent (five or ten minutes in the case of muscle). They are then rapidly washed in water, brought for about twenty minutes into 1 per cent. gold chloride solution, washed again in water, and brought into a bottle containing 50 c.c. of distilled water and 2 drops of acetic acid. They are exposed to the light, and the reduction is complete in twenty-four or forty-eight hours. The preparations thus obtained are good for immediate study, but are not permanent on account of their over-blackening with time, the reduction of the gold being incomplete. In order to obtain perfectly reduced, and therefore permanent, preparations, the reduction should be done in the dark in a few cubic centimetres of dilute formic acid (1 part acid to 4 of water). The reduction is complete in twenty-four hours.

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[1] 'Quart. Journ. Mic. Sci.' (N.S.), lxxx (1880), p. 456.

[2] 'Traité,' p. 826.

[3] 'Traité,' p. 813.

115. Gold Chloride (*Viallanes' method*[1]).—The tissues are treated with osmic acid (1 per cent. solution) until they begin to turn brown, then with one-fourth formic acid for ten minutes; they are then put into solution of chloride of gold of 1·5000 (or even much weaker) for twenty-four hours in the dark, then reduced in the light in one-fourth formic acid.

116. Gold Chloride (*Gerlach's method*[2]).—Spinal cord is hardened for fifteen to twenty days in a 1 to 2 per cent. solution of bichromate of ammonia. Thin sections are made and thrown into a solution of 1 part of double chloride of gold and potassium to 10,000 parts water, which is very slightly acidulated with HCl. They remain there from ten to twelve hours, and having become slightly violet are washed in hydrochloric acid of 1 to 2 : 3000 strength, then brought for ten minutes into a mixture of 1 part HCl to 1000 parts of 60 per cent. alcohol, then for a few minutes into absolute alcohol, and thence into clove oil.

117. Gold Chloride and Arsenic (*Manfredi's method*[3]).—Tissues previously hardened in 2 per cent. solution of bichromate of potash are put for half an hour into solution of arsenic acid, or into 1 per cent. acetic acid. They are then put into 1 per cent. gold chloride for half an hour, washed in water, and reduced in sunlight in 1 per cent. arsenic acid solution, which is changed for fresh as fast as it becomes brown. Mount in glycerin. Sunny weather is necessary.

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[1] 'Hist. et. dév. des Insectes,' 1883, p. 42.

[2] 'Stricker's Handb.,' p. 678 (1872).

[3] 'Archivio per le scienze mediche,' vol. v, No. 15.

118. Gold Chloride and Oxalic Acid (*Manfredi's method*[1]).—Fresh tissues are treated as follows: Gold chloride, 1 per cent., half an hour; oxalic acid, 0·5 per cent.; they are then warmed in a water-bath to 36°, allowed to cool, and examined. Mount in glycerin. Sunny weather is necessary.

119. Gold Chloride and Pritchard's Solution (*Carrière's method*[2]).—Formic acid (50 per cent.) until the tissues are transparent; gold chloride, 1 per cent., twenty minutes; water; reduce in Pritchard's solution from midday till next morning in the dark; wash with water, treat with alcohol, and imbed in paraffin if sections are to be made.

Pritchard's solution is composed of amyl-alcohol 1 per cent., formic acid 1 per cent., water 98 per cent.

120. Chloride of Gold and Cadmium (*Ciaccio's method*[3]).—Lemon-juice five minutes. Wash in water. Double chloride of gold and of cadmium, 1 per cent., half an hour (in the dark); wash in water; formic acid, 1 per cent. (in the dark), twelve hours; expose to sunlight twelve hours; undiluted formic acid twenty-four hours, in the dark; wash in water. Mount in glycerin.

For corneæ and other parts rich in nerves.

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[1] 'Archivio per le scienze mediche,' vol. v, No. 15.

[2] 'Arch. Mik. Anat.,' xxi (1882), p. 146.

[3] 'Journ. de Microgr.,' vii (1883), p. 38. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 290.

CHAPTER XII.

OSMIUM, CHLORIDES OF PALLADIUM AND OF IRON, PRUSSIAN BLUE, SILVER NITRATE.

121. Osmic Acid.—Osmic acid has the property of staining all fatty matters very rapidly and of a deep black. This property renders it available for the study of the distribution of medullated nerve-fibres, which it stains of a deep bluish black. It is frequently recommended in the books for the study of nerve-endings, for which it is of next to no use (*quâ* its staining properties). For the study of the distribution of nerves, whether medullated or non-medullated, gold chloride is the most fitting reagent; for nerve-endings it is infinitely superior to osmic acid. Osmic acid is also frequently recommended as a general stain. This recommendation proceeds from the fact that osmic acid will in course of time, and that not a very long time, stain all kinds of tissue that are submitted to its action, colouring them, according to the duration of exposure to it, of all shades of bluish or brownish grey down to jet black. But here again it must be said that this is a misleading recommendation, and that osmic acid should only be used as a general stain when it is not convenient or possible to employ one of the tried selective stains in general use. Its action is most uncertain. Sometimes it gives a diffuse stain in which nuclei are brown, and protoplasm and formed tissue steely grey; sometimes the tissue and protoplasm is grey, whilst nuclei are unstained, appearing as empty spaces in the protoplasmic network. It is next to impossible so to control the action of the acid as to obtain the desired degree of colouration. It has very little penetrating power, so that different parts of a preparation are generally stained by it with very unequal intensities. It does not readily combine with other reagents so as to form a double stain. It hinders subsequent staining with carmine, and if used after a carmine stain generally destroys it. It will, of course, be understood that the objections here set forth have regard to the employment of osmic acid as a stain; it is a very poor stain, but I am fully alive to its admirable qualities as a fixing agent.

It is best applied in the form of vapour. The preparations should be exposed to the vapour of the solid acid, or of a strong solution, until the desired colouration is obtained. If fresh tissues be so treated they may be made to afford a good nuclear stain with carmine afterwards, that is, if they have not taken up too much of the metal. This too is the best way of treating tissues that have already been stained with carmine, if it is desired to preserve the carmine stain.

Of solutions, a plain aqueous solution may be used, or combinations of this with glycerin, or with chromic acid or bichromate of potash solution. In any case a weak percentage of osmic acid should be taken, in no case more than 0·5 per per cent.

Small specimens of fresh tissues may remain, for instance, for fifteen to thirty minutes in a 0·5 per cent. aqueous solution, and then be *well washed out* either with water or alcohol, and further treated as may be desired. The smaller and more permeable Crustacea sometimes give instructive preparations when treated in this way. For delicate, easily-permeable structures, much weaker solutions should be taken. Small Medusæ and Ctenophora may be made to furnish instructive preparations by treatment for a few minutes with a solution of about 0·1 per cent. or less.

For staining the tissues of vertebrates the glycerin or bichromate of potash solution should, I think, be preferred.

Tissues may be immersed in a solution of one-third glycerin, or a mixture of glycerin 1 part, alcohol 1 part, water 1 to 3 parts, to which about 1 per cent. of a 1 per cent. solution of osmic acid has been added and left there for several days until sufficiently stained.

Osmic-acid staining. Subsequent treatment of the tissues.—In order to prevent the blackening with time that generally happens to tissues that have been treated with osmic acid, several methods are available. The best is probably bleaching by free chlorine (Mayer's method, No. 476), or the specimens may be treated with ammonia carmine or picro-carmine, or they may be put for some hours into solution of bichromate of potash or chromic acid. This is a very good plan. Whitman recommends treatment for twenty-four hours with Merkel's solution (No. 17), which actually bleaches the tissues if blackened ('Journ. Roy. Mic. Soc.,' (N.S.) iii, p. 912). I find the method gives excellent results.

The best carmine stain for osmium objects is, I think, alum-carmine. Borax-carmine or hæmatoxylin may also be used. Picro-carmine is generally recommended, but frequently results in a very dirty stain.

122. Osmic and Chromic Acid Staining Combination (*Max Flesch's formula*) :

Osmium	0·10 parts.
Chromic acid . . .	0·25 ,,
Water	100.00 ,,

For twenty-four to thirty-six hours. Is a good fixing and hardening agent. It gives a general stain of very little precision, and is certainly not to be recommended as a stain. The same is the case with Flemming's chromo-aceto-osmic mixture, No. 14.

123. Bichromate of Potash and Osmium.—By adding a little osmium and about one-fourth of glycerin to Müller's solution, a good hardening fluid is obtained, which gives, I think, a better stain than Max Flesch's formula. A still better stain is obtained by treating for twenty-four hours with osmic acid (of 0·1 per cent. in dilute glycerin) specimens that have been hardened in bichromate of potash.

124. Osmic and Oxalic Acid (*Brösicke's method*[1]).—Osmic acid, 1 per cent., one hour. Wash out the acid carefully, and put for twenty-four hours into cold saturated aqueous solution of oxalic acid.

Most kinds of tissue are stained by this treatment of various hues of carmine, the nuclei being darker. The tissues are well preserved. Care must be taken that the tissues do not become blackened before removal from the osmic-acid bath, as in that case the oxalic acid is powerless to redden them. (I have tried this stain and find it diffuse.)

125. Palladium Chloride.—Weak solution of palladium chloride (1·500, 1·800, 1·000) should be taken and allowed to act on the tissues for several days. An irregular general stain, varying in the different tissues from yellow to inky black (medullated nerve) is obtained.

Not to be recommended as a stain, but admirable as a hardening agent.

For the manner of preparing the solution and other details *see* HARDENING AGENTS, No. 215.

126. Prussian Blue Impregnation (*Leber's method*[2]).—Treat the tissue for a few minutes with $\frac{1}{2}$ per cent. solution of sulphate of protoxyde of iron, and then with 1 per cent. solution of red prussiate (ferricyanide) of potassium. Wash with water.

127. Perchloride of Iron Staining Process (*the Hoggans' method*[3]).—This method was first published, three years before the date quoted, in the 'Journal of the Quekett Club.'

The tissue (having first been fixed with $\frac{1}{2}$ per cent. silver nitrate, which is somewhat reduced by a short exposure to diffused light) is dehydrated in alcohol, and treated for a few minutes with 2 per cent. solution of perchloride of iron in spirit. It is then treated with a 2 per cent. solution of pyrogallic acid in spirit, and in a few minutes more, according to the depth of tint required, may be washed in water and mounted in glycerin.

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[1] 'Science Gossip,' No. 175 (1879), p. 160. 'Journ. Roy. Mic. Soc.,' ii (1879), p. 764.

[2] Ranvier, 'Traité.' p. 108.

[3] 'Journ. Roy. Mic. Soc.,' ii (1879), p. 358.

The process is not applicable to chromic acid or bichromate specimens. This method has been re-invented by Fol, with the difference that the iron perchloride is employed by him as a fixing agent (see No. 35). It gives better results than any other for the study of Tintinnodea (*vide* 'Arch. Sci. Phys. et Nat.,' ix, 1883, p. 554, and 'Journ. Roy. Mic. Soc.,' (N.S), iii (1883), p. 730).

The method may be recommended as being in many respects an excellent one.

128. Silver Nitrate.—Nitrate of silver is an *interstitial-staining* agent, employed for the purpose of demonstrating the *contours* of cells, *intercellular spaces*, fine lymph *canals* and *lacunæ* in tissues. This reagent was first suggested by Coccius, but was brought into vogue by the observations of His and Recklinghausen.[1] The general principles of its employment are so well stated by Ranvier[2] that I cannot do better than abstract his account.

Silver nitrate may be employed either in solution or in the solid state. The latter method is the less frequently employed, but is easy and gives good results. It is useful for the study of the cornea and of fibrous tissue, but is not suitable for epithelia. For the cornea, for instance, proceed as follows. The eye having been removed, a piece of silver nitrate is quickly rubbed over the anterior surface of the cornea, which is then detached and placed in distilled water; it is then brushed with a camel's-hair brush in order to remove the epithelium. The cornea is then exposed to the action of light. On subsequent examination it will be found that the silver nitrate which was dissolved by the liquid that bathes the surface of the cornea, has traversed the epithelium and soaked into the fibrous tissue, on the surface of which it is reduced by the action of light. The cells of the tissues will be found unstained.

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[1] See *Recklinghausen*, "Zur Geschichte der Versilberungsmethode," in 'Virchow's Arch.,' xxvii (1863), p. 419.

[2] 'Traité,' p. 105.

Silver nitrate is generally employed in solution in the following manner: A 1 per cent. solution is taken, to which 2, 3, or 4 volumes of water are added according to circumstances. The mode of employment varies in its details according to circumstances, a point which it is very important to observe. In the case of a membrane such as the epiploon, the membrane must be stretched like a drum-head over a porcelain dish, and washed with distilled water in order to remove the albuminates and white blood-corpuscles that are found on its surface; it is then washed with the solution of silver nitrate. In order to obtain a powerful stain it is necessary that this part of the operation be performed in direct sunlight, or at least in a very brilliant light. As soon as the tissue has become white and has begun to turn of a blackish grey, the membrane is removed, washed in distilled water, and mounted on a slide in some suitable examination medium.

If the membrane were left in the water, the cells would become detached and would not be found in the finished preparation.

If the membrane had not been stretched as directed, the silver would be precipitated not only in the intercellular spaces, but in all the small folds of the surface, and the forms of the cells would be disguised.

If the membrane had not been washed with distilled water before impregnation, there would have been formed a deposit of silver on every spot on which a portion of an albuminate was present, and these deposits might easily be mistaken for a normal structure of the tissue. It is thus that very often impurities in the specimen have been described as stomata of the tissue. . . .

If the solution be taken too weak, for instance, 1·500 or 1·1000, or if the light be not brilliant, a *general* instead of an *interstitial* stain will result; nuclei will be most stained, then protoplasm, and the intercellular substance will contain but very little silver.

In general, in a good "impregnation," the contents of cells, and especially nuclei, are quite invisible.

Ranvier notes that when tissues are to be impregnated by immersion, they should be constantly *agitated* in the silver-bath in order to avoid the formation on their surfaces of deposits of chlorides and albuminates of silver which would give rise to deceptive appearances.

Impregnation with silver may be followed by treatment with picro-carmine (or other carmine stain[1]), which will bring out the nuclei provided the impregnation has not been overdone.

It should be noticed that impregnations only succeed with *fresh* tissues, and cannot be made to succeed with tissues preserved in any way.

129. Silver Nitrate Staining Solutions (*Ranvier's formulæ*[2]).—The solutions generally employed by Ranvier vary in strength from 1·300 to 1·500. Thus 1·300 is used for the epiploon, pulmonary endothelium, cartilage, tendon, whilst a strength of 1·500 is employed for the study of the phrenic centre, and for that of the epithelium of the intestine. For the impregnation of the endothelium of blood-vessels (by injection) solutions of 1·500 to 1·800 are taken.

130. Silver Nitrate Staining Solutions (*Duval's formulæ*[3]).—Solutions of 1, 2, or at most 3·100, are recommended.

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[1] Except ammonia-carmine, the free ammonia of which would quickly dissolve-out the black impregnation-lines.

[2] 'Traité.'

[3] 'Précis,' &c., p. 229.

In order to prevent the over-blackening of the preparations, which very generally happens in the course of time, it is recommended that the silver be fixed by plunging the tissues, as soon as impregnated, for a few seconds into a bath of hyposulphite of soda (2 per cent.), after which they must be washed in distilled water as usual.

131. Silver Salts Staining Solution (*Alferow's formulæ*[1]).—Duval states that Alferow recommends the soluble silver salts of organic acids, viz. the picrate, lactate, acetate, and citrate, as giving better results than the nitrate. He employs them in solutions of 1·800, and adds to the solution employed for staining a small quantity of the acid of the salt taken (10 to 15 drops of a concentrated solution of the acid to 800 c.c. of the solution of the salt). The object of the free acid is to decompose the precipitates formed by the action of the silver salt on the chlorides, carbonates, and other substances existing in the tissues, leaving only the albuminate, which is a more resistant compound.

132. Silver Nitrate Staining Solution (*Tourneux and Hermann's formula*[2]).—In their fine studies on the epithelia of invertebrates, these authors employed a solution of 3·1000 strength, and in some cases weaker solutions. The tissues were allowed to remain in the silver-bath for one hour, and were washed out with alcohol of 36° strength.

133. Silver Nitrate Staining (for lymphatics of pancreas) (*the Hoggans' method*[3]).—The tissue having been stretched is treated with 1 per cent. silver nitrate solution "quickly poured on and off," washed, and the silver reduced by excessive exposure to light. This may be followed by staining with hæmatoxylin.

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[1] 'Arch. de Physiol.,' 1874. 'Laboratoire d'histologie du Collège de France,' 1874, p. 258. Duval, 'Précis,' p. 230.

[2] Robin's 'Journal de l'Anat.' (1876), p. 200.

[3] 'Journ. of Anat. and Physiol.,' xv (1881), p. 477.

CHAPTER XIII.

COAL-TAR COLOURS IN GENERAL.

134. The following "Classified List of the chief Anilin Dyes, with their Solubilities in Water and in Spirit" (page 106) is taken from a paper by Dr V. Harris in 'Quart. Journ. Mic. Sci.' (N.S.) xc (1883), p. 301.

135. Coal-tar Colouring Matters. Anilin Stains.—It would be out of place here to attempt an exposition of the extremely complicated chemistry of the coal-tar colours, and the reader is referred for information on that subject to the treatises on the chemistry of dyeing and of the carbon compounds in general. (The following books are recommended: Bolley, 'Die chemische Technologie der Spinnfasern, &c., Fortgesetzt von Kopp and Meyer,' and Schultz, 'Die Chemie des Steinkohlentheers,' &c., Braunschweig.)

It is more important for us to note those general properties of the anilin dyes that have relation to their applicability to histological staining processes. The great advantages offered by the majority of these dyes are that they stain most animal tissues almost instantaneously and with great brilliancy, whilst the stain readily takes effect on tissues hardened in chromic acid, which latter is, as we shall see, in certain cases a very important point. A decided disadvantage attaching to the whole group is the doubtful permanence of the stain, and a further disadvantage of most of them is that the stain is extracted by alcohol, or by the clearing media,

Brown.	Red.	Orange.	Yellow.	Green.	Blue.	Violet.
Bismarck—partially sol. in water; sol. in dilute spirit. **Vesuvin**—sol. in water. **Chrysoidin**—sol. in water.	**Eosin**, Pink—freely sol. in water. **Anilin Scarlet**—insol. in water; freely so in methylated spirit. **Flamingo**, deep brownish red—partly sol. in water; freely so in meth. spirit. **Ponceau**,[1] deep red crimson—partly sol. in water; freely in dilute spirit. **Rosanilin**—partly sol. in water; freely sol. in dilute spirit. **Fuchsin**—partly sol. in water; sol. in dilute spirit.	**Aurin**—insol. in water; partly sol. in strong spirit; more so in absolute alcohol. **Anilin Orange**—ditto, ditto. **Tropeolin**, in deep yellow glistening scales—partly sol. in water; more so in meth. spirit. **Phosphin**, yellowish orange—partially sol. in water; more so, but not freely, in spirit. **Safranin**—sol. in water and in spirit.	**Fluorescin**, greenish yellow—insol. in water; sol. in spirit, the solution being beautifully fluorescent. **Anilin Primrose**—only partly sol. in meth. spirit.	**Iodine Green**, blue green—freely sol. in water or spirit. **Malachite Green**, a less blue green—freely sol. in water and in spirit.	**Soluble Anilin Blue**—freely sol. in water. **Bleu de Lyon**—insol. in water; freely so in strong spirit. **Methylen Blue**, a very deep blue—freely sol. in water and in spirit. **China Blue**—freely sol. in water. **Serge Blue**—ditto. **Blue Black**—freely sol. in water.	**Hoffman's Violet**—freely sol. in water and in dilute spirit. **Methyl Violet**, the red predominating—sol. in water partially; freely sol. in spirit. **Gentian Violet**, the blue predominating—freely sol. in water. **Tyrian Blue**, near to violet—sol. in water. **Spiller's Purple**—sol. in spirit.

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[1] Ponceau is a mixture of rosanilin and phosphin.

thus rendering necessary special precautions for the preservation of stained specimens. Very few of these dyes naturally give a nuclear stain, so that such a stain can only be obtained by means of a special method of washing out—the Böttcher-Hermann process, of which, as explained and modified by Flemming, I proceed to give an account. This is followed by an account of Griesbach's examination of various anilin colours not studied by Flemming, and that, again, is followed by special paragraphs devoted to some other important anilin stains, which will conclude this part of the matter; the subject of *double staining* being treated separately.

From an examination of the literature abstracted in this chapter it must be concluded that very few of the anilin stains at present known are capable of being useful to the morphologist—to the pathologist they may be of far greater importance. At Naples only two anilins are generally employed, viz. Bismarck brown and safranin. The worker at morphology will probably find the following stains sufficient for all his needs, viz. as nuclear stains, iodine green, methyl green, Bismarck brown, safranin; whilst anilin blue is sometimes useful as a diffuse stain for rendering visible very delicate membranes, transparent chitinous structures and the like, either as a single dye, or used after a carmine stain to produce a double stain.

136. Anilin Staining for Nuclei (*Böttcher's and Hermann's method*[1]).—I take the following from a paper by Flemming, l. c.:

It was made out independently by Böttcher and by Hermann that by washing out with alcohol the diffuse stain of anilin or "nitro-colours," clearing with oil of cloves, and mounting in balsam, a fine *nuclear* and *permanent* stain may be obtained. That which makes this method peculiarly valuable is that it enables us to obtain permanent preparations of nuclei brilliantly and precisely stained in tissues that have been fixed with *chromic acid.* For the study of cell structure and cell processes chromic acid is one of the most valuable of fixing agents, but is defective in that it is incompatible with precise staining by means of the usual colouring matters, such as carmine and hæmatoxylin ; it becomes at once much more valuable now that we are able to employ it without sacrificing the power and precision of stain that is so important. The method is further precious in that it affords the only means known of preserving anilin-stained preparations, which (except in certain ill-defined cases) lose their colour in glycerin and in acetate of potash. The first hint of the process appears to be due to Böttcher (*vide* 'Reichert u. Du Bois-Reymond's Archiv,' 1860, p. 373, and Virchow's 'Archiv,' Bd. xlix, p. 302).

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[1] 'Arch. Mik. Anat.,' xix (1881), pp. 317 and 742.

Böttcher's process.—He treated his preparations as follows: Müller's solution followed by alcohol, staining with nitrate of rosanilin (dissolved in glycerin and water), washing out with alchohol, clearing with *kreasote*, mounting in balsam. Flemming has two criticisms on this form of the method; the first, that Müller's solution does not preserve nuclei in their true forms, the second that kreasote attacks the stain.

Hermann's process.—Hermann's process was first published in the 'Tagblatt der Grazer Naturforschersammlung,' 1869, p. 105.

It consisted in hardening in alcohol tissues either previously fixed with chromic acid or fresh, staining in a concentrated alcoholic solution of fuchsin, and further treating as above mentioned. He afterwards succeeded with other anilin colours, especially safranin and rose de naphthaline.

137. Flemming's Methods.—Flemming's modifications of the method are as follows:

The preparations are fixed in chromic acid (of from 0·1 to 0·5 per cent., according to the nature of the tissue). They may remain in it, so far as their staining is concerned, for a few hours, or for months; but it is better not to leave them for more than a few weeks, lest they become brittle. If alcohol be employed to harden after the action of the acid, this must first be washed out with water; and the alcohol should be taken weak at first.

For staining, only sections, or very thin and permeable portions of tissues must be taken. They must be washed free from the acid in water. They are then stained for twelve to twenty-four hours in a small quantity (about 1 c.c.) of a solution of one of the under-mentioned colours in absolute alcohol, diluted about one half with water; except in the case of *dahlia*, which is best employed in aqueous or acetic-acid solution without alcohol. They are then rinsed with alcohol, and brought into absolute alcohol for half a minute or longer (until they take on a *translucent* colouration). They may then either be cleared and mounted at once, or put into distilled water whilst they are being looked over and sorted. To mount them, they are cleared with oil of cloves and mounted *at once* in dammar; they must be mounted at once, because oil of cloves extracts the colour. Kreasote does so in a still greater degree.[1] Specimens that have been treated with alcohol instead of chromic acid, and that have lain too long in it, no longer take on a nuclear stain with anilin colours.

Flemming has experimented with the following colours:

Safranin.
Magdala red (Rose de Naphthaline).
Dahlia.
Mauve (mauvëin) (which is the chloride of a base allied to the base of safranin).
Rouge fluorescent (which has a similar composition to the last).
Solid green (which is the oxalate of a base obtained by oxidation of Fischer's leukobase or tetramethyltri-

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[1] On this point see *infra*, **Clearing Agents.**

phenylmethan; analogous in constitution to malachite green).

Ponceau (which is the sodium salt of the acid compound dinitroxylolsulphate of naphthol).

Orange (which is the sodium salt of the acid compound dinitrobenzolsulphate of diphenylamine).

Eosin. Fuchsin. Bismarck brown.

Most of these colours were obtained from the manufactory of Bindschedler u. Busch, *Basel.*

Eosin and ponceau were found to be useless, as they cannot be made to afford a nuclear stain. Orange stains precisely, but too weakly. All the others give good results. Mauvëin and rouge fluorescent often stain some nuclei much more deeply than others in the same preparation. Solid green gives a weaker stain than safranin and magdala. It is specific as regards the "Kerngerüst" and nucleoli. Fuchsin gives a weaker stain than magdala, safranin, dahlia, and mauvëin. Bismarck brown was not very satisfactory with chromic acid preparations; but with fresh alcohol preparations gave a good, though not quite pure, nuclear stain. The best results were in general obtained with *safranin*, *magdala*, and *dahlia*.

The chief modification of Hermann's method insisted on by Flemming is, *prolonged* staining in *strong* solutions.

Flemming sums up as follows the advantages of this process:—"*Whenever it is desired to preserve in their true aspects the structure of nuclei, and the figures of nuclear division, as they are preserved by means of chromic acid pre-eminently;* and further, *to make such structures and figures susceptible of minute study by means of strong and accurate staining, in all such cases this method deserves preference over all others.* Where, however, no more is desired than a general nuclear stain without regard to minute faithfulness of fixation, other known methods are more convenient, especially *alcohol-alum-carmine.*"

The method may be applied to objects fixed with osmic acid, and (still better) osmium-chromic acid (Flesch's formula, No. 13).

138. Griesbach's Experiments.[1]—Griesbach examined in respect of their applicability to histological technic the colours mentioned in the following list. They were most of them obtained from Herrn Dr E. Nölting, Director of the School of Dyeing at Mulhouse (*Chemieschule für chemische Farbenindustrie*).

The colouring matters in question are mostly deep-hued, representing all shades of yellow, orange, and red. The shades are distinguished by the capital letters—Y (yellow), O (orange), R (red), OO, RR, &c.

Anilins studied by Griesbach.[2]

Commercial name.	Chemical name.	Obtained from—	Literature.
1. Anilin yellow (Anilingelb)	Amidobenzol	Any large manufactory	Jahresber. d. prakt. Chem., 1861, 82, 462.
2. Saüregelb or Echtgelb	Amidoazobenzol-sulfosäure	Ibid.	Chem. Industr., 1879, 49, 346.
3. Chrysoidin	Diamidoazo-benzol	Williams, Thomas, and Dower, Brentford and Fulham, near London, or as No. 1	A. W. Hoffman, Berl. Ber., 1877, 10, 213; N. O. Witt, ibid., 1877, 10, 550, 654; Griess, ibid., 1877, 10, 388.
4. Vesuvin (Phenylenbraun, Bismarckbraun, Manchester-braun)	Triamidoazo-benzol	Dr. E. ter Meer and Comp. chem. Farbenlabr. Uerdingen a. Rh.	Ztsch. f. Ch., 1867, N.F., 3, 278.
5 Tropaeolin Y.	Phenolazobenzolsulfosaures Natrium	Durand and Hugenin, Basel	Ber. d. d. Chem. Ges., 1879, 259.

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[1] 'Arch. Mik. Anat.,' xxii, p. 132.

[2] Ibid., p. 134.

Commercial name.	Chemical name.	Obtained from—	Literature.
6. Tropaeolin O, Chrysoin, Chryseolin, Tropaeolin R	Resorcinazobenzolsulfosaures Natrium	Ter Meer u. Co.	Witt, Privatmitth. an Griess u. Griess in Ber. d. d. Chem. Ges., 1878, 2143.
7. Tropaeolin OO, (Orange IV, Orange N)	Diphenylaminazobenzolsulfosaures Kalium	Ibid.	Bericht der d. chem. Ges., 1879, 259.
8. Tropaeolin OOO, Nr. 1 (Orange I)	α Naphtolazobenzolsulfosaures Kalium	Durand u. Hugenin, Basel	Ibid.
9. Tropaeolin OOO, Nr. 2 (Orange II, Chrysaurein, β Naphtol-orange)	β Naphtolazobenzolsulfosaures Kalium	Ibid.	?
10. Crocein	Azobenzolsulfosäure-ammonium azo β naphtolsulfosaures Natrium	Farbenfabriken vorm. Friedr. Bayer u. C. in Elberfeld	D. R. P., Nr. 18027, 18th March, 1881.
11. Aechtroh Roccellin (Orseillin, Nr. 3, Rubidin, Rauracienne)	β Naphtolazonaphtalinsulfosäure	Badische Anilin u. Sodafabr. in Mannheim. Or, Durand u. Hugenin, Basel	?
12. Ponceau R aus Salz R[1] (Xylidin-Ponceau)	Xylolazo β naphtoldisulfosäure	Fabr. von Meister, Lucius u. Brüning. in Höchat a. M.	D. R. P., Nr. 3229, 24 April, 1878; Chem. Indust., 1878, 410.
13. Ponceau RR aus Salz R[1]	Pseudocumolazo β naphtoldisulfosäure	Ibid.	Ibid.
14. Ponceau G aus Salz G[1]	Xylolazo β naphtoldisulfosäure	Ibid.	Ibid.
15. Ponceau GG aus Salz G[1]	Pseudocumolazo β naphtoldisulfosäure	Ibid.	Ibid.

Commercial name.	Chemical name.	Obtained from—	Literature.
16. Bordeaux R aus Salz R[1]	Naphtalinazo-βnaphtoldisulfosäure	Ibid.	Ibid.
17. Bordeaux G aus Salz G[1]	Same as 16	Ibid.	Ibid.
18. Biebricher Scharlach (Ponceau RRR[1])	β Naphtolazobenzolsulfosäurenatriumazobenzolsulfosaures Natrium	Aktiengesellschaft für Anilinfabrikation in Berlin	Bericht d. d. chem. Ges., 1880, 801; ibid., 1880, 542, 803, 980.
19. Orange III (Helianthin, Gold-Orange)	Dimethylanilinazobenzolsulfosaures Ammonium	Ter Meer u. Co.	?

The following are the practical results obtained from Griesbach's examination:

Anilin yellow.—1. Insoluble in water, soluble in alcohol. Useless.

"*Säuregelb*" *or* "*Echtgelb.*"—2. Soluble in water. Bone stains a good orange, cartilage and connective tissue citron-yellow. Fresh and alcohol preparations stain well, not so chromic-acid ones. A nuclear stain, but somewhat diffused into the plasma. Good differentiation of the different tissues in a large section, ranging from brightest citron to brown. Use concentrated solutions, as the stain is considerably washed out by alcohol.

Chrysoidin.—3. Soluble in water. A diffuse yellow stain; does not stain bone or any sort of connective tissue; does not answer with alcohol or chromic-acid preparations.

Bismarck brown (*Vesuvin*).—4. A nuclear stain, both for alcohol and chromic-acid preparations; most suitable for unicellular organisms, bacteria, leucocytes, &c. The stain is not easily washed out by alcohol.

Tropæolin Y.—5. Soluble in water. Fresh connective tissue and muscle of marine invertebrates are not stained, aquatic forms stain a weak citron; alcohol-preserved marine mollusca behave like fresh ones, chromic-acid hardened spinal cord is unstained, gland tissue diffusely; decalcified bone put up in alcohol stains well. Is not washed out by alcohol.

Tropæolin O.—6. Dissolve in boiling water and filter. Bone stains deep orange, connective tissue, muscle, and cartilage yellowish, epithelium brownish, gland tissue yellow; chromic-acid preparations, especially nerve-centres, citron yellow. Is not washed out by alcohol.

Tropæolin OO.—7. (Same as Flemming's orange, except that Flemming employed the sodium salt.) Soluble in water. Resembles the action of Tropæolin O, but seems to be a weaker stain. Not washed out by alcohol.

Tropæolin OOO, No. 1.—8. Soluble in water. Bone and connective tissue dark orange; both fresh and alcohol and chromic-acid preparations stain well. A fine nuclear stain, no-wise diffuse, more powerful than Tropæolin OO. Very resistant to alcohol.

Tropæolin OOO, No. 2.—9. Soluble in water. Stains in the same way as No. 1, but is very easily washed out by alcohol. Should be useful for the Hermann-Flemming's nucleus-staining method.[1]

Crocein.—10. Soluble in water. Stains (purple-red) bone, cartilage, connective tissue and muscle (both fresh and alcohol). Very good results with glandular tissues (pancreas, liver, testicle). Chromic-acid objects stain well. A very useful stain.

Rocellin.—11. Soluble in water. A cherry-red stain for bone, connective tissue, muscle, glandular tissue, epithelia; alcohol and chromic-acid objects stain well. A nuclear stain for the Hermann-Flemming's process. Easily washed out by alcohol. Bone sections gave the best results.

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[1] 'Tageblatt der deutsch Naturforschergesellschaft in Graz,' 1875, p. 105.

Xylidinponceau.—12. Soluble in water. Stains well alcohol preserved bone, connective tissue, and muscle; glandular tissue diffusely; chromic-acid objects not stained. Washed out by alcohol.

Ponceau RR.—13. Soluble in water. Not fit for chromic-acid objects, but in other respects preferable to Xylidinponceau, as being a more precise nuclear stain. Resists alcohol.

Ponceau G.—14. Soluble in water. Bone stains deep orange, connective tissue, muscle, and epithelia saffron yellow. A nuclear stain for glandular tissues. Chromic-acid objects do not stain; better for vertebrates than for invertebrates. Washed out by alcohol, except from bone.

Ponceau GG.—15. Soluble in water. Useless for chromic-acid objects. Stains (sharp orange) bone, connective tissue, and muscle, useless for other tissues. Washed out by alcohol.

Bordeaux R.—16. Dissolve in warm water to which a few drops of alcohol have been added. Bone, muscle, and connective tissues a fine Bordeaux red; alcohol objects better than fresh ones, chromic-acid objects stain. A nuclear stain, very useful for glandular tissues. Resists alcohol.

Bordeaux G.—17. Dissolve in warm water. Same reactions as Bordeaux R, but a somewhat yellower tone.

Biebricher Scharlach.—18. Soluble in water. Stains all tissues (alcohol) uniformly bright red. Not good for chromic-acid objects, nuclei are brought out sharply. Resists alcohol.

Gold orange.—19. Soluble in water. Stains both fresh, alcohol, and chromic-acid preparations; bone orange red, muscle and cartilage golden, connective tissue reddish. A most excellent stain for glandular tissues (kidney, prostate, injected liver—Berlin blue injection). Use strong solutions as the stain is somewhat washed out by alcohol.

In working with any of the above stains be careful in the matter of using acids or alkalies subsequently, as they often cause change of colour or throw down precipitates in the tissues.

Dehydrate, clear with an ethereal oil and mount in balsam. Oil of cloves is recommended. (If the hue of the stain be so delicate as to run any risk of being masked by the yellow of the clove oil, use instead lavender or an absolutely colourless oil of aniseed. In this case be on the look-out to avoid shrinkage and brittleness arising in the tissues.)

CHAPTER XIV.

IODINE- AND METHYL-GREEN, ANILIN-BLUE, QUINOLÉIN, INDULIN, METHYL-VIOLET, SAFRANIN, BISMARCK BROWN, EOSIN, BENGALE ROSE.

139. Iodine-Green ("Hoffmann's Grün") (*Griesbach's method*[1]).—Iodine-green, or Hoffman's green, is the hydriodide of tetramethylrosanilinmethyliodide. As to the chemistry of the substance, see Hofmann and Girard 'Ber. chem. Ges.,' ii, 440, Hofmann, *ibid*, vi, 552, and Hofmann, 'Monatsbericht der Königl. Academie der Wiss. zu Berlin,' 15th July, 1869.

Griesbach employs the following solution:

Crystallised iodine-green . .	0·1 gr.
Distilled water . , . .	35·0 ,,

These proportions may be varied according to the desire of the operator, within limits indicated only by the observation that good results can only be obtained from deep-hued solutions.

The objects are to be put into water for a few seconds before staining. They stain instantaneously in general. They are to be washed out in water, and brought into glycerin, or dehydrated in absolute alcohol and passed through oil of cloves or anise-seed into balsam or dammar. *The stain is not destroyed by immersion in alcohol for days.* The preparations are apparently permanent in balsam.

Alcoholic solutions may be used for staining, but Griesbach finds no advantage in so doing.

A nuclear but frequently diffuse stain, valuable for the exceeding rapidity of its action, and for its striking power of marking-out by staining in various hues the different forms of tissue. For instance, in a section through the uterus of a roe-deer, the epithelia are stained blue, the glands dark green, and the muscle-fibres malachite green, whilst the connective tissue remains unstained.

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[1] 'Zool. Anz.,' No. 117 (vol. 5, 1882), p. 406.

In general bone and connective tissue do not take the stain, gland-cells stain most intensely and selectively, muscle stains instantaneously and diffusely, but the nuclei are brought out by their deeper colouration, the sarcolemma remaining unstained. Good nuclear stains are obtained with blood, spermatozoa, and bacteria (*sic*).

It is useful for ganglion-cells and for axis cylinders. In sections of spinal cord, and most especially in sections of skin, it affords most instructive preparations. In sections of kidney instructive differentiations are obtained. *Chromic-acid preparations stain well.*

On the whole, Griesbach states that "in many respects iodine-green performs decidedly more than all other anilin colours employed in histological practice." "It is the most useful of all anilin stains."

This colour is somewhat expensive to prepare, and for this reason is no longer found in commerce, having been superseded by methyl-green. But the high price is no impediment to the use of iodine-green in histology, on account of the small quantity of the substance required for staining.

The presence of iodine may be tested in the following way: A little of the solid colouring matter is treated with sulphuric acid, and a few small fragments of bichromate of potash are added; the iodine, if present, escapes in the form of violet vapours. It may also be demonstrated by means of chloroform or sulphide of carbon.

The colour may be obtained of excellent quality from C. A. F. Kuhlbaum's Chemische Fabrik, Berlin, S. O.[1] ('Zool. Anz,' No. 130 (1883), p. 56.)

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[1] Dr. Harris found that both malachite and iodine-green are "not at

140. Methyl-Green (*Calberla's observations*[1]).—Calberla first obtained this substance in 1874 from the "Chemische Fabrik" of M. B. Vogel in Leipzig. It then went by the name of "Vert en cristaux."

He then found that "the nuclei of subcutaneous connective tissue and those of vessels and nerve-sheaths stained rose red, cells of the corium reddish white, and the cells of epidermis greenish blue to pure blue." Observations made in 1876 with fresh samples of methyl-green, gave in the main the same results, but the effects were not invariably produced and the stain was not always stable.

141. Methyl-Green (*Griesbach's method*[2]).—Methyl-green is not a derivate of rosanilin, but is obtained by treating methyl-violet with methyl-nitrate (instead of methyliodide, as in the manufacture of iodine-green).

Griesbach employs it in the same way as iodine-green; it gives tolerable preparations, but these cannot be compared to those obtained by means of iodine-green, and are less resistant to alcohol.

Viallanes employs this reagent for the study of the histology (principally nervous) of insect larvæ. The tissues are treated with 1 per cent. solution of acetic acid, and stained (on the slide) in an aqueous solution of methyl-green ('Recherches sur l'histologie des Insectes, etc.,' Thèse, Paris, 1883.)

142. Methyl-Green (*Carnoy's methods*[3]).—Methyl-green has been largely employed by Carnoy for the study of nuclei in the *fresh* state. He finds that it is a pure nuclear stain. He states that it has the further advantage of being an admirable fixing agent for nuclei. Cells die in it instantaneously, and

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all permanent," *vide* 'Quart. Journ. Mic. Sci.,' 1883 (N.S.), No. xc (p. 300).

[1] 'Morph. Jahrb.,' iii (1877), 3 Hft., p. 625.

[2] 'Zool. Anz.,' 117 (1882), p. 410.

[3] Carnoy, 'La Biologie Cellulaire,' pp. 92, 144.

preserve their form for many hours. He uses a tolerably concentrated aqueous solution, to which it is frequently advisable to add 1 per cent. of acetic acid (glacial) or a trace of osmic acid (0·1 to 1 per cent.). Washing out is easily done with water.

143. Methyl-Green (for amyloid degeneration) (*Curschmann's method*[1]).—"A 1 per cent. aqueous solution is used, a few minutes' immersion being sufficient; a more uniform colouration is produced by using a more dilute solution and immersing the section for a longer time. Alcohol, turpentine, and oil of cloves quickly discharge the colour, hence specimens cannot be mounted in balsam (*sic*), but may be mounted in glycerin."

"Dr. Curschmann, of Hamburg, claims that methyl-green has a peculiar affinity for amyloid substance, colouring it an intense violet; surrounding tissues that have not undergone degeneration are stained green or bluish green. The contrast is striking; the smallest spot of amyloid disease can be readily discovered. Methyl-green also colours hyaline casts ultramarine blue, so in a section of the kidney the healthy tissue would appear green, hyaline casts blue, and amyloid spots violet."

144. Picro-Anilin (*Tafani's method*[2]).—Tafani was led to seek for a *green* stain, obtained by the combination of anilin-blue with picric acid, through observing that blue stains in general are (optically) unfavorable to definition. Picric acid was chosen in preference to chromic acid on account of its greater harmlessness to the tissue-elements, whilst anilin-blue was taken as the blue colouring agent on account of its more selective action on certain tissues (spleen, lymphatic, central nervous tissues), and its resistance to acids.

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[1] 'Louisville Medical Herald,' ii (1880), p. 123. 'Journ. Roy. Mic. Soc.,' iii (1880), p. 857.

[2] 'Journ. Roy. Mic. Soc.,' i (1878), p. 82. 'Journal de Micrographie.'

The solutions of picric acid and anilin-blue may be employed separately or mixed. In either case the solutions should be *saturated.* Take, for instance, of a saturated picric-acid solution 100 vols., and anilin-blue solution 4 vols. A few minutes suffice for staining preparations of the lymphatic glandular system.

If it be desired to employ the two solutions separately, use the *blue first,* and allow the preparations to remain in it until they have acquired a light sky-blue tint. This will give a nuclear stain. The preparations should then be passed for fifteen minutes into the picric-acid solution, the result being a *green nuclear stain.*

Tissues that have been treated with hardening reagents, such as chromic acid, will take this stain.

The stained tissues may be treated with *weak* acids (acetic, carbolic, hydrochloric) without harm; alkalies should be avoided. Preparations may be mounted in fluids or in balsam. I understand the author to say that glycerin affects the colour in time. The alcohol used for dehydrating, or the glycerin used for mounting, should be slightly tinged with picric acid or the colour will be washed out.

The operation *may be abridged* in the case of balsam-mounting by simply dehydrating the specimens after staining in the anilin-blue, by means of alcohol containing ½ per cent. of picric acid.

145. Parma-Blue (*Frey's method*[1]).—Parma-blue (which is obtained by treating diphenyl-rosanilin with sulphuric acid) gives when dissolved in water (say 1·1000) a fine violet blue solution. Tissues stain in this in a few minutes. Rinse with water and mount in glycerin or dehydrate with alcohol and mount in balsam.

146. Anilin-Blue (*Heidenhain's formula*[2]).—Take an aqueous solution of such concentration that a watch-glassful appears of a forget-me-not tone when placed on a white ground. "It must be neutral. If a drop of acetic acid be added, or even if it be exposed to vapour of acetic acid, the solution takes on a much deeper hue, and its staining powers are enhanced. Ammonia on the contrary discolours it. . . . Into a watch-glassful of the above solution (4 c.c. in content) I bring about a dozen alcohol-hardened sections. They remain there for twenty-four hours, in a closed space saturated with vapour of water in order to prevent the concentration of the solution. Mount the stained sections, and cement the mounts at once in glycerin; for if they are left in excess of glycerin exposed to the air, the stain is gradually drawn out, which does not happen when the air is at once excluded."

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[1] 'Arch. Mik. Anat.,' iv (1868), p. 346.

[2] 'Arch. Mik. Anat.,' vi (1870), p. 404.

I have translated the directions literally, and must leave the reader to make out whether Heidenhain means to direct him to take a neutral or a slightly acid solution. Probably he means that neutral solutions are to be employed, and precautions taken to prevent the access of acetic-acid vapour. But why?

147. Quinoléin-Blue (*Ranvier's method*[1]).—The quinoléin should be dissolved in alcohol of 36° strength, and the solution diluted with an equal volume of water. (If the alcohol were taken dilute in the first instance, the blue would not dissolve.) The solutions employed for staining should be very weak as the quinoléin stains very powerfully.

After staining, wash and mount in glycerin. When first mounted, nuclei will be seen to be stained a fine violet, nerves of a grey blue, smooth muscle blue, protoplasm blue, fat deep blue. But after twenty-four hours in the glycerin, the aspect of the preparation is changed; the nuclei have become colourless; the protoplasm remains blue, and is seen to contain granulations stained intensely blue; nerves remain grey blue, but frequently contain granulations stained blue. Quinoléin, in a word, has the property of staining fatty matters an intense blue.

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[1] 'Traité technique' (1875), p. 102.

If the stained preparations be treated with solution of potash of 40 per cent. strength, the differential reaction is produced immediately; the nuclei are unstained, protoplasm, nerve, and muscle tissue are pale blue, and fatty matters deep blue.

148. Cyanin or Quinoléin-Blue (*Certes' method*[1]).—Cyanin or quinoléin-blue is imperfectly soluble in water; but for the purpose for which this dye is recommended, viz. the staining of infusoria, a very weak solution suffices. An aqueous solution of $\frac{1}{600,000}$ is strong enough. If the solution be made with common filtered water the infusoria are not destroyed, but continue to live for from twenty-four to thirty-six hours. Distilled water must be avoided if it be wished not to kill the infusoria, as it is a quick poison for these organisms.

Lymph-corpuscles may be stained during life by means of a solution of cyanin in serum.

In the case of infusoria, the reaction is peculiar in so far as the stain is limited to the "fatty granulations of the protoplasm." It is very feeble in the sarcodic expansions, in the cilia, the cuticle, and the contractile vacuoles; the nuclei and nucleoli are completely free from it. Certes considers that cyanin is one of the best tests for fatty matters.

In order to stain dead infusoria, Certes kills the animals in a solution of cyanin in one-third alcohol (the strength of the solution is 1 : 100,000). The reaction in this case is different from that described above; certain elements, and sometimes the nucleus, are stained violet, and very various colourations are sometimes observed in one and the same preparation. Certes was not able to determine the conditions of these reactions.

Cartilage and cellulose stain violet.

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[1] 'Comptes rendus' (1re série), 1881, p. 425, and 'Zool. Anz.,' No. 81 (1881), p. 209, and ibid., No. 84, p. 288.

The stain is in no case permanent in glycerin.

149. Indulin (*Calberla's observations*[1]).—Indulin dissolves into a dark-blue solution in warm water or in dilute alcohol. For staining, the concentrated aqueous solution should be diluted with six volumes of water. Sections will stain in the dilute solution in five to twenty minutes; they may be washed in water or in alcohol and examined either in glycerin or oil of cloves.

The peculiarity of this stain is that it never stains nuclei; the remaining cell-contents and intercellular substance are stained blue. In its general effects it resembles quinoléin-blue, and is exactly the opposite of methyl-green. The stroma of tendinous tissue, for instance, stains of a fine blue, the connective tissue that surrounds the bundle, hardly at all, and the tendon-corpuscles of Ranvier remaining perfectly colourless stand out as white stellate figures on a blue ground.

150. Anilin-Violet (*Orth's method*[2]).—Sections are to be soaked in water, and then brought into the following solution:

Anilin-violet	1 part.
Acetic acid	300 parts.

Mount, without washing out, but simply draining, in acetate of potash (acetate 2 parts, water 1 part).

The stain will probably fade within a year or two.

151. Safranin (*Pfitzner's formula*[3]).—One part safranin dissolved in 100 parts absolute alcohol; after a few days 200 parts of distilled water are added.

An excellent stain for nuclei, being purely nuclear. Stains quickly. Works best with chromic-acid preparations from which the acid has been removed as far as possible.

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[1] 'Morph. Jahrb.,' iii (1877), 3 Hft., p. 627.

[2] 'Amer. Mon. Micr. Journ.,' i (1880), p. 143. 'Journ. Roy. Mic. Soc.' (N.S.), i (1881), p. 137.

[3] 'Morph. Jahrb.,' vi, p. 478, and vii, p. 291. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 878.

According to Whitman ('Journ. Roy. Mic. Soc.', l. c.), this stain is in use at Naples.

152. Anilin-Violet or Methylanilin (*Weiss's methods*[1]).—Iodine, iodated chloride of zinc, and sulphate of indigo, are well-known tests for amyloid substances. To these in late years has been added anilin-violet, which possesses the property of staining of a more or less lively red the regions affected with amyloid degeneration, whilst other tissues are stained of an intense blue.

152a. Safranin.—To all of these, Weiss prefers *safranin.* Safranin is perfectly soluble in alcohol, imperfectly so in water; stains tissues in a few instants rose, nuclei more intensely than cell substance. Both fresh and alcohol-hardened tissues are well stained; less well those that have been hardened with chromic acid. Preparations keep well in a saturated solution of acetate of potash. As regards testing for amyloid substances, the reaction is as follows: healthy tissues stain of a fine rose colour, those affected with amyloid degeneration of a fiery orange yellow.

The preparation employed by the author goes by the name of "rose safranin."

153. Methyl-Violet (Methylanilin=anilin-violet=Paris-violet=inchiostro di Leonardi) (*Capparelli's experiment*[2]).—Referring to the above-mentioned dichroic reaction of anilin-violet, Capparelli, suspecting it to be an optical and not a chemical reaction, examined unstained sections by light transmitted through a thin layer of anilin-violet, and found the reaction was produced just as if they had been stained, that is, the amyloid substance appeared red, and the healthy tissues violet. He obtained the same effect with some other dichroic liquids. He then mounted sections of affected organs on glass stained with the seven colours of the spectrum, and found that *on the violet glass amyloid matter appears red, whilst normal tissues were of a deeper violet-like hue.* From these experiments he deduces that it is a property of amyloid substances to stop the violet rays whilst letting the red rays pass. (Spectroscopic examination proved that anilin-violet contains these two sets of rays.)

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[1] 'Archivio perle scienze mediche,' iii, No. 14 (1879), p. 2.

[2] 'Archivio perle scienze mediche,' iii, No. 21, p. 1.

154. Bismarck Brown (*Weigert's formula*[1]).—"Bismarck brown" is an anilin colour prepared by the Berlin "Actiengesellschaft für Anilinfarben-fabrikation." It may be used in weak alcoholic or concentrated aqueous solution. The latter is prepared by boiling the colour in water. Filter the solution. (It must be refiltered from time to time.)

A precisely nuclear stain. Alcohol or chromic-acid preparations are deeply stained in a few moments in the concentrated solution; in a few minutes in weaker solutions. Wash out in absolute alcohol if it be desired to employ oil of cloves and Canada balsam; or first in water and then in alcohol if glycerin be chosen. Does not overstain, and does not overwash out. Is selective for "plasma-cells" and for many forms of bacteria and micrococcus. May be combined with other colours to produce double stains. The colour of the stain is brown, a fact which gives it a special importance where it is desired to photograph preparations.

155. Bismarck Brown (*Mayzel's formula*[2]).—Bismarck brown is dissolved in acetic acid. The preparations may be studied in glycerin, but soon fade. Used by Flemming and others for the study of nuclei.

156. Bismarck Brown (*Mayer's formula*[3]).—According to Whitman, whose report is here given, Mayer uses a saturated solution of Bismarck brown in 70 per cent. alcohol.

157. Eosin.—The eosin of commerce is stated by Fischer, who was, I believe, the first to introduce this substance into histological practice, to be the potassium salt of tetrabromide of fluorescein. ('Arch. Mik. Anat.,' xii (1875), p. 349.) (M. Duval calls it the potassium salt of a bromide of phthaléin, the *primerose* of commerce.) It is soluble both in alcohol and in water. By treating an aqueous solution with acids (*e.g.* HCl), the free colouring matter is precipitated, and may be employed for staining in alcoholic solution (see below).

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[1] 'Arch. Mik. Anat.,' xv (1878), p. 258.

[2] 'Arch. Mik. Anat.,' xviii (1880), pp. 237, 250.

[3] 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 878.

Eosin staining solutions have great penetrating power, and produce a powerful rose-coloured stain in a very short time. A few years ago they were highly in favour in many laboratories; but there are serious objections to their use, arising from the diffuseness of the stain and the difficulty of getting stained preparations to keep in the usual mounting media. It is probable that hereafter this reagent will only be used in combination with some other staining material, either with the view of ensuring greater penetration (*e.g.* Lang's picro-carmine and eosin), or to obtain a double stain (as in the case of hæmatoxylic eosin). Eosin-stained preparations must not be treated with acids; they should be mounted in neutral, or better, saline glycerin containing 1 per cent. of NaCl and charged with a little eosin (otherwise the colour would diffuse out from the preparations); or in balsam after dehydrating with alcohol and clearing with clove oil, both similarly charged with eosin.

158. Aqueous Eosin Staining Solution (*Fischer's formula*[1]).—An aqueous solution of commercial eosin of 1 : 10 or 1 : 20 strength may be made, and a few drops of it added to a watch-glass of water in which are the objects to be stained. These may remain in the solution for ten or twelve hours, when they may be washed out with water or alcohol. A diffuse stain. For mounting, see the last paragraph.

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[1] 'Arch. Mik. Anat.,' xii (1875), p. 349.

159. Alcoholic Eosin (*Fischer's formula*[1]).—If an aqueous solution of eosin be treated with acids, the *free colouring matter* is thrown down, and may be separated by filtration, and dissolved in 20 to 30 parts of alcohol (absolute alcohol is best). A few drops of this solution are added when required for staining to a watch-glass of alcohol.

The alcoholic solution of the free eosin colouring matter is generally preferable to the aqueous solution of the potassium salt, and especially so in the case of preparations that have been hardened in Müller's solution. For in this case the chromic acid of the bichromate of potash throws down the free colouring-matter of the eosin in the form of a red precipitate that can only be kept soluble in concentrated alcoholic solution. If it is desired to use the solution of the potassium salt, therefore, the chromic acid must first be neutralised by addition of some alcali.

Both these solutions are stable. Fresh preparations stain better in the alcoholic than in the aqueous solution. The axis cylinder of nerves stains deep rose-red, whist the myelin is hardly coloured at all. Muscle stains deeply. Like the first, a diffuse stain.

160. Alum-Alcoholic-Eosin.—Free eosin 1 part, alum 1 part, absolute alcohol 200 parts. Not superior to the latter formula.

161. Ammonia-Eosin (*Lavdowsky's formula*[2]).—Eosin is dissolved in ammonia, which is allowed to evaporate, and the solution is then diluted with water. It should be neutral or very feebly alkaline. One or two drops of this are diluted with water until the mixture appears barely coloured in a watch-glass placed on a white background. The sections are placed in this watch-glass and removed therein to a desiccator (or placed under a glass shade) containing a watch-glass of dilute acetic acid (one to two drops of the concentrated acid to 3 c.c. of water), and left exposed to the vapour of the acid for twenty-four hours.

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[1] 'Arch. Mik. Anat.,' xii (1875), p. 349.

[2] 'Arch. Mik. Anat.,' xiii (1876), p. 359.

The ammonia-eosin may also be used in a more concentrated solution, in the ordinary way.

162. Picro-Eosin (*Lavdowsky's formula*[1]).—To an ammoniacal solution of eosin that has been exposed to the air for two or three days, concentrated picric acid is added to neutralisation. The solution may be used in divers degrees of concentration.

Both these solutions of eosin stain better than that of Fischer's formula (No. 158), since eosin is not sufficiently soluble either in water or in alcohol. The ammoniacal solutions do not stain diffusely, as is the case with Fischer's solutions.

163. Bengal Rose (*Griesbach's method*[2]).—Bengal rose, or "Rose bengale," or "Bengal rosa," is an eosin dye. Griesbach states that it is a chloride of the tetriodide of fluorescin, and belongs to the Resorcin phtaléins. It is the bluest of the eosin dyes as yet known, approaching in hue to fuchsin, but possessing far greater brilliancy and purity of hue. In aqueous solution it is very useful for staining chromic-acid objects, especially spinal cord, in which the grey matter stains of a deep bluish-red, and stands out boldly from the less deeply coloured white matter. Connective tissues and muscle both of vertebrates and invertebrates take the stain well. It is not useful for bone or glandular tissues as these stain diffusely, and the stain is too much extracted from them by alcohol. It is very useful for double and treble stains, as will be explained below.

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[1] 'Arch. Mik. Anat.,' xiii (1876), p. 359.

[2] 'Zool. Anz.,' No. 135 (1883), p. 172.

CHAPTER XV.

BACTERIA-STAINING.

THE processes that it is necessary to employ for the staining of Schizomycetes are of a somewhat special nature, so that it appears desirable to give here a special account of them.

164. Bacteria-Staining (*Blanchard's method*[1]).—A film of bacteria from an infusion is fixed by treating it with strong osmic acid on a slide. It is then covered, the osmic acid drawn off, and a drop of violet of methylanilin run in under the cover. In half an hour's time the preparation may be completed by running in glycerin to which is added a small quantity of the violet in order that the stain may not be extracted from the organisms. Or concentrated solution of sulphate of calcium may be used instead of glycerin. The bacteria are stained of a fine violet, the ground-substance remaining colourless.

Other anilin stains may be used, but methyl-violet appears to be the most durable.

Hæmatoxylin may be used. In this case the film of bacteria should be stained in it for twenty-four hours (after fixing with osmic acid); the iridescence which is then formed and spoils the clearness of the preparation is removed by repeated washing, and the membrane is mounted in glycerin or chloride of calcium.

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[1] 'Rev. Inter. Sci.,' iii (1879), p. 245. 'Journ. Roy. Mic. Soc.,' ii (1879), p. 463.

165. Bacteria-Staining (*Weigert's methods*[1]).—Weigert remarks that most *micrococci* may be coloured by means of the ordinary nuclear stains; *e.g.* Schweigger-Seidel's carmine, anilin or hæmatoxylin. They are coloured red by all the nuclear carmine stains, by purpurin, by fuchsin, and by magdala red; brown by Bismarck brown and vesuvin; brownish-violet by carmine, if the preparation be subsequently washed with tincture of sesquichloride of iron; green by methyl-green; blue and violet by hæmatoxylin, iodine-violet, methyl-violet, dahlia, and gentian-violet. The anilins are used by overstaining in strong aqueous solutions, and washing out with alcohol or acetic acid, or both.

For the larger *bacilli*, only anilins can be used; carmine and hæmatoxylin are useless. The stains specially recommended are, Bismarck brown, methyl-violet, methyl-green, safranin, fuchsin, magdala, and gentian-violet, the last being most recommended. It is employed in a 1 per cent. aqueous solution. Wash out for an hour in alcohol and afterwards put up in water, alcohol, or oil of cloves. In the case of sections of pathological tissues, the nuclei of the tissues may subsequently be stained with carmine, cochineal, alum-carmine, borax-carmine, or picro-carmine.

166. Methylen-Blue with Vesuvin (for staining the bacillus of tuberculosis) (*Koch's method*[2]).—This is the process by which Koch first demonstrated the bacillus of tuberculosis. A thin layer of tubercular material is spread over a cover-glass, "dried, and warmed for a few moments over a flame, so as to render it insoluble; it is then placed for twenty-four hours in a mixture of 1 c.c. of concentrated solution of methylen-blue in alcohol, 0·2 c.c. of 10 per cent. solution of potash, and 200 c.c. distilled water. After the twenty-four hours the preparation is found to be coloured blue; a few drops of a solution of vesuvin are then placed on it, which has the effect of discharging the methylen-blue from all the tissue-elements, but not from the bacilli. The former are of a brown colour, and the blue bacilli are conspicuously defined." The preparation is finished with absolute alcohol, oil of cloves, and balsam. "This peculiarity of being rendered visible by the combined action of methylen-blue and vesuvin is possessed only by the tubercle-bacilli and by those of leprosy. All other bacteria and micrococci known to Koch lose, under the action of vesuvin, the blue colour which they acquire from methylen-blue."

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[1] 'Arch. f. Path. Anat.,' lxxxviii (1881), p. 275. 'Journ. Roy. Mic. Soc. (N.S.), i, p. 838.

[2] 'Verh. Physiol. Ges. Berlin' (1882), p. 65. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 385.

167. Staining Bacilli of Tuberculosis (*Ehrlich's method*[1]). The alkali used in Koch's process "exercises a modifying action on the different histological elements and on the bacteria themselves. . . . Ehrlich therefore sought for another base, acting in a less powerful manner, and found it in *phenylamin* or *anilin*."

A thin layer of expectorated matter is spread on a cover-glass, dried, and fixed by warming for an hour at 100° or 120° C., or by passing rapidly four or five times through the flame of a spirit lamp. The cover is then floated with the tubercular layer downwards, for a quarter to half an hour on the surface of the staining fluid, which is prepared as follows:

"A saturated solution of phenylamin is to be made in distilled water, by shaking with the water the excess of anilin which floats on it, and carefully filtering the whole. To the transparent liquid thus obtained add, drop by drop, a saturated alcoholic solution of fuchsin or methyl-violet until a slight opalescence is produced." This is the first part of the process. The preparation must now be washed out, so as to leave the bacteria alone stained. The cover is therefore floated on to nitric acid diluted with two volumes of water; nitrous vapours are at once disengaged, and the preparation becomes absolutely colourless in a few seconds. Mount in balsam. "Under the microscope the bacteria are seen to be very clearly coloured red or violet; but by reason of their extreme delicacy they often escape the eye and require the most accurate focussing. It is therefore better to study them in preparations which have been slightly coloured blue or green (when fuchsin has been used for the first bath), or yellow (when methyl-violet has been used)."

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[1] 'Bull. Soc. Belg. Micr.,' vii (1882), p. cxvii. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 573.

168. Anilin and Methyl-Violet for Tubercle-Bacteria (*Van Ermengen's method*[1]).—Instead of making (as in Ehrlich's process, *vide supra*) a solution of anilin in water, which only takes up 1 part in 30, an alcoholic solution is made (4 grammes of liquid anilin in 20 grammes of alcohol of 40°, adding an equal quantity of distilled water, and filtering before use). Van Ermengen found that the most stable colouring agents were sulphate of rosanilin and methyl-violet—B B B B B. Decolourise in dilute nitric acid, and wash in distilled water.

169. Potash Process for Tubercle-Bacteria (*Baumgarten's method*[2]).—A film of tuberculous matter is spread on a cover-glass, which is then placed in a watch-glass and covered with distilled water, to which is added some drops of a 33 per cent. solution of caustic potash. Without any further preparation the bacteria may then be recognised under a power of 400 to 500, particularly if a light pressure is applied to the cover-glass so as to disengage them more completely from the detritus which surrounds them.

If it be wished to distinguish them more clearly from other bacteria, the preparation should be dried by passing the cover-glass rapidly two or three times through a flame, and stained with concentrated aqueous solution of anilin-violet or other colour. The bacteria of tuberculosis are *absolutely colourless*, while the other bacteria, micrococci, &c., are plainly coloured. The whole process only takes ten minutes.

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[1] 'Bull. Soc. Belg. Micr.,' vii (1882), p. cli. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 706.

[2] 'Centralbl. f. d. Med. Wiss.,' 24th June, 1882. 'Journ. Roy. Mic Soc.' (N.S.), ii (1882), p. 706.

170. Magenta and Chrysoidin for Tubercle-Bacteria (*Heneage Gibbes's method*[1]).

℞ Magenta crystals	2 grammes.
Pure anilin	3 „
Alcohol (sp. gr. ·830)	20 c.c.
Distilled water	20 c.c.

Dissolve the anilin in the spirit, rub up the magenta in a glass mortar, adding the spirit gradually until it is all dissolved, then add the water gradually, while stirring, and keep in a stoppered bottle.

A thin layer of sputum is spread on a cover-glass, dried, and fixed by passing the cover two or three times through the flame of a small Bunsen burner. It is then floated, with the sputum-layer downwards, on the surface of a small quantity of the magenta solution, where it remains for fifteen or twenty minutes. Wash in dilute nitric acid (33 per cent.), until all colour has disappeared: wash out the acid with water, when a faint colour will return; float on to the surface of saturated solution of chrysoidin in distilled water; let the preparation remain for a few minutes until it has taken on the brown stain; wash out with water; place in absolute alcohol for a few minutes; remove and dry perfectly in the air; mount in balsam.

In order to preserve the aqueous solution of chrysoidin, there should be added to it a crystal of thymol dissolved in a little absolute alcohol. Both the magenta solution and the chrysoidin solution should be *filtered* into the watch-glass in which the staining is performed.

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[1] 'Lancet,' ii (1882), p. 183. 'Journ. Roy. Mic. Soc.' (N.S.) ii (1882), p. 895.

171. Rosanilin and Methyl-Blue for Tubercle-Bacteria (*Heneage Gibbes's method*[1]).—Take of rosanilin hydrochloride 2 grammes, methyl-blue 1 gramme; rub them up in a glass mortar. Then dissolve anilin oil 3 c.c. in rectified spirit 15 c.c.; add the spirit slowly to the stains until all is dissolved, then slowly add distilled water 15 c.c; keep in a stoppered bottle.

The sputum having been dried on a cover-glass in the usual manner, a few drops of the stain are poured into a test-tube and warmed. As soon as steam rises, pour into a watch-glass, and place the cover-glass on the stain. Allow it to remain for four or five minutes, then wash in methylated spirit until no more colour comes away; drain thoroughly and dry, either in the air, or over a spirit lamp; mount in balsam.

The stain can be used cold equally well, but in that case the cover-glass must be left on it for at least half an hour.

The bacilli of tubercle are stained red, micrococci and bacteria blue. The results are "very satisfactory, and the horrible nuisance of the nitric acid is avoided."

171a. Gentian-Violet and Iodine, for Schizomycetes in Tissues (*Gram's method*[2]).—A solution of gentian-violet is prepared according to the formula of Ehrlich (*supra*, 167), and the sections, after having been soaked in absolute alcohol, are stained in it from one to three minutes (except in the case of tubercular bacilli, which require twelve to twenty-four hours). They are then placed in a solution composed of iodine 1 gramme, iodide of potassium 2 grammes, water 300 c.c. After three minutes therein they are brought into absolute alcohol (in some cases it is better to immerse them in absolute alcohol *before* the iodine bath, as well as after it).

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[1] 'Lancet,' i (1883), p. 771. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 764.

[2] 'Fortschr. d. Medicin,' ii (1884), No. 6. 'British Med. Journ.,' Sept. 6, 1884, p. 486. 'Journ. Roy. Mic. Soc.' (N.S.), iv (1884), p. 817.

Change the alcohol once or twice so as to well wash out the sections, clear in clove oil (which completes the decolouration) and mount in balsam. A double stain, the bacteria being violet and the tissue-elements faint yellow. The colour of the latter may be deepened by immersing the sections for *a moment* in saturated aqueous solution of vesuvin, after they have been transferred from the iodine solution to alcohol. A *longer* immersion in the vesuvin would cause the violet colour to be extracted from the bacteria. Dehydrate with alcohol and mount as before.

The following schizomycetes take this stain: those of crupose pneumonia, of pneumonia, of the liver abscesses after perityphlitis, of circumscribed infiltration of the lungs, of osteomyelitis, of arthritis suppurativa after scarlatina, of nephritis suppurativa after cystitis, of multiple brain abscesses, of erysipelas, of tubercular cattle distemper, and those of putrefaction.

171b. Methylen-Blue and Acetic Acid.[1]—To 100 parts of solution of caustic potash of 1 : 10,000 add 30 parts of saturated alcoholic solution of methylen-blue. Filter. Stain for one or two hours, wash out with acetic acid of ½ per cent., followed by water. Dehydrate with absolute alcohol, clear with oil of *cedar*, and mount in balsam. This is specially recommended for bacteria of glanders, typhoid fever, and some others; and it is stated to be also the most universally successful stain for bacteria *in tissues* in general.

171c. Gentian-Violet and Vesuvin (*Weigert's method*[2]).—Take of a 2 per cent. aqueous solution of gentian-violet 12 c.c., and of a saturated aqueous solution of anilin oil 100 c.c. Mix. Stain sections in the usual way. Then stain for 15 minutes in the following solution: Bismarck brown, 1 gr.; spiritus vini rectificati (sp. gr. ·830), 10 c.c.; distilled water,

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[1] 'British Med. Journal,' Sept. 6, 1884, p. 486.

[2] 'Practitioner,' xxxiii (1884), p. 35. 'Journ. Roy. Mic. Soc.' (N.S.), iv (1884), p. 818.

100 c.c. For tubercle bacilli in sections of tuberculous growths. Klein states that the results are very beautiful, both with fresh and with hardened tissues; but the stain is liable to fade.

171d. Fuchsin and Oxalic Acid (*Hartzell's method*[1]).—A cover of sputum having been prepared and fixed by heat in the usual way is stained for from three to five minutes in Gradle's fuchsin solution, washed with water, and decolourised by saturated solution of oxalic acid, washed, dried, and mounted in glycerin or balsam. The bacilli (of tuberculosis) are stained of a brilliant red, and no staining of the background is necessary,

Gradle's fuchsin solution is prepared as follows: carbolic acid 15 minims, distilled water ½ fluid oz., dissolve, and add saturated alcoholic solution of fuchsin ½ fluid dr.

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[1] 'Journn. Roy. Mic. Soc.' (N.S.), iv (1884), p. 652.

CHAPTER XVI.

MULTIPLE STAINS—WITH CARMINE AS A NUCLEAR STAIN.

172. Of late years considerable attention has been paid to the problem of staining the various elements of a preparation in various colours, in order that by this means the optical differentiation of the tissues may be enhanced. Some useful formulæ have been settled; but it should be borne in mind that the usefulness of all, or almost all, multiple stains is very restricted. For the making of didactic preparations I believe that most of the formulæ lately proposed may be very useful; for the purpose of discovering new anatomical facts I believe that most of them are well-nigh useless. It may be quite true that it may be convenient to be able to show to a class of students a section of the aorta stained with picric acid and picro-carmine, and to be able to explain it to them concisely by saying, "The red fibres are connective tissue, the yellow fibres are elastic tissue, and the brown fibres are smooth muscle;" but it is also true that a preparation made so as to give such results (picric acid, twenty-four hours; make sections, stain picro-carmine, mount in Farrant's medium[1]) would possess but slight advantages, with very considerable disadvantages, if it were proposed by means of it to investigate the structure of connective tissue, or of yellow elastic tissue, or of smooth muscle, for the first time; or if it were proposed by means of it to make some new addition to our knowledge of these structures. I know that in England of late years a great many sections of young rats' tails and children's larynges have been polystained in many different colour combinations; but I am not aware that any new fact of scientific importance has been brought to light by any of these methods. I hold that those cases in which it is desirable to employ more than one stain are rare; and that of the many formulæ for multiple staining, very few are useful for anatomical discovery.

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[1] Stirling, in 'Journ. of Anat. and Physiol.,' xv (1881), p. 351.

I distinguish two classes of multiple stains. In the one a pure nuclear stain, taking effect on *all* the nuclei of *all* the tissues of a preparation, is combined with a stain taking effect on *all the extra-nuclear parts of all the tissues.* Borax-carmine followed by indigo-carmine is a typical example of such a combination. In the second class a stain taking effect on the totality of the elements of any one tissue exclusively is combined with a stain or stains of another colour taking effect on the totality of the elements of the other tissues. In this class, nuclear staining is generally neglected, polychroism being frequently, it would appear, the chief thing aimed at; in this case the preparations have at most the value of diagrams, and frequently have no other use than to be sold or exchanged. The first class, aiming at enhancing the usefulness of a pure nuclear stain by improving the definition of extra-nuclear parts, has a legitimate scientific end in view, and is capable of rendering service in research.

173. Picro-Carmine.—Picro-carmine is a double stain, if care be taken not to wash out the picrin beyond the point desired. And it is one of the best of double stains. *See ante* Nos. 64, *et seq.*

174. Borax-Carmine and Picro-Carmine.—A very beautiful and precise double stain may be obtained by means of this combination. I add to a watch-glassful of Grenacher's alcoholic carmine a few drops of picro-carmine mixed with alcohol to the strength of the borax-carmine. It is sometimes well to further dilute the mixture of the two stains with 70 per cent. alcohol, and to stain very slowly (twenty-four hours). Wash out with HCl in alcohol as in the case of borax-carmine alone. Nuclei should appear pure carmine-red, protoplasm orange, formed tissues generally very pale yellow. For *Appendicularia* I find this stain gives better results than any other. And I found the same with *Sagitta*.

Prof. Fol informs me that he obtains a similar stain by washing out borax-carmine objects with HCl-alcohol in which a little picric acid is dissolved. The HCl is taken *weaker* in this case.

174a. Borax-Carmine and Indigo-Carmine (*Merkel's method*[1]).—The following is from a paper read by Golding-Bird before the "Medical Microscopical Society" in April, 1877 describing the method originated by F. Merkel in Germany in 1874, and from another account, l.c. xviii, p. 242.

(A) Take half a drachm of carmine, two drachms of borax, and four ounces of water. Rub up in a mortar, allow the fluid to stand some time, decant, filter, and keep in a stoppered bottle.

(B) Take two drachms indigo-carmine, two drachms borax, and four ounces water. Mix, decant, filter, and preserve, as before.

Before using, mix A and B in equal proportions.

The objects to be stained must be thin; all traces of chromic acid or chromates must have been carefully washed out from them; and they must be soaked in alcohol before staining. Stain for fifteen or twenty minutes. Wash out with saturated aqueous solution of oxalic acid, for a rather shorter time; wash the acid out with water, and mount as desired.

The oxalic acid is necessary for fixing the indigo-carmine, which being very soluble in water would otherwise be washed out. Unfortunately, it precipitates carmine, so that successful preparations are not easily obtained; the carmine being generally either precipitated or turned into a straw colour. Marsh ('Section Cutting,' p. 85) speaks of this process as having given him the best results of all double stains tried by him.

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[1] 'M. M. J.,' xvii (1877), p. 317.

175. Borax-Carmine and Indigo-Carmine (*Seiler's method*[1]).—Stain in Woodward's borax-carmine (No. 74), wash out in HCl one part, alcohol four parts, until the sections assume a bright rose colour (which appears in a few seconds). Wash the acid out of the sections, and stain for six to eighteen hours in a mixture of two drops of sulphindigotate of soda solution with one ounce of 95 per cent. alcohol. The mixture should be filtered before using (No. 103).

Nuclei red, formed material slightly tinged with blue. Connective-tissue fibres deep blue, blood-vessels purplish. "Epithelium and hair take this staining in a very curious manner, inasmuch as the cells of different ages take different colours, ranging from a brilliant emerald-green to purple-violet and olive-green, thus affording a valuable means of differentiation, especially in epitheliomas, where the so-called pearls are brought out with general distinctness, being of a different colour from the rest of the cells."

Permanent. An excellent process.

It is obvious that this method may be modified, in most cases with advantage, by using Grenacher's alcoholic borax-carmine (No. 81) instead of the aqueous solution of Woodward. Heneage Gibbes uses the borax-carmine quoted under his name, No. 75.

176. Carmine and Anilin-Blue (*Duval's method*[2]).—Stain with carmine "in the ordinary way;" dehydrate; and stain for a few minutes (ten minutes for a section of nerve-centres) in an alcoholic solution of anilin-blue (ten drops of saturated solution of anilin-blue soluble in alcohol to ten grammes of absolute alcohol, for sections of nerve-centres). Clear with turpentine and mount in balsam.

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[1] 'Am. Quart. Mic. Journ.,' i (1879), p. 220. 'Journ. Roy. Mic. Soc.,' ii (1879), p. 613.

[2] 'Précis de technique microscopique,' 1878, p. 225.

The sections should appear of a fine dark violet when taken from the anilin; they are extremely transparent under the microscope. Nerve-cells and axis-cylinders, reddish violet; blood-vessels, bluish violet, and so sharply marked out that the preparations have the aspect of injections. The connective elements are stained of a nearly pure blue, so that it is easy to distinguish them from the nervous elements.

Applicable to all kinds of tissues, but especially to sections of nerve-centres.

177. Picro-Carmine and Iodine-Green (*Stirling's method*[1]). —Stain picro-carmine, wash in acidulated water (acetic acid), stain iodine-green. Iodine-green stains very rapidly, and care must be taken not to overstain. Rinse in water, dehydrate *rapidly*, clear with clove oil, *mount in dammar*. (All preparations stained with iodine-green *must* be mounted in dammar.)

Iodine-green has a specific action on adenoid tissue and mucous glands, which it stains of a bright green.

Applications.

Fœtal cancellous bone.—(Section of head of bone.) All the newly-formed bone is red, but in the centre of each of the osseous trabeculæ the residue of the calcified cartilage on which bone is deposited is stained green.

Ossifying articular cartilage.—(Vertical section of cartilage and subjacent bone where the epiphysis is united to the shaft, but where the line of ossification still exists.) The articular cartilage and the remains of the calcified cartilage trabeculæ on which the new bone is deposited, *green*.

Posterior part of tongue : mucous and serous glands.—Mucous glands *green*, serous glands red, adenoid tissue green.

Peyer's Patch.—(Section of small intestine of dog or cat, hardened in a mixture of 2 parts of a $\frac{1}{8}$th per cent. solution of chromic acid and 1 part of methylated spirit, for two weeks, stain as above.) All adenoid tissue and mucous glands green, connective tissue bright red, muscularis mucosæ light brown.

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[1] 'Journn. Anat. and Physiol.,' xv (1881), pp. 349, *et seq.*

Solitary glands of large intestine.—Ut supra.

Trachea.—(Section.) Mucous glands and cartilage green, connective tissue red, trachealis muscle yellowish brown.

Bronchus, same effect.

Skin.—(Preferably from the sole of the foot of a fœtus.) Cuticle and superficial layers of epithelium yellow, rete Malpighii green, ducts of sweat glands green, connective tissue of cutis vera red.

Cerebellum.—Outer layer of grey matter with cells of Purkinje red, inner or granular layer green.

178. Picro-Carmine, Anilin-Green, and Malachite-Green (*Richardson's method*[1]).—Richardson operated by placing picro-carmine stained sections in "watery solutions of iodine and malachite-green dyes in different proportions until they seemed dark blue in colour."

When equal parts of the two solutions were taken the nuclei of cartilage-cells became light purplish grey, the newly-formed osseous walls of the cancellous tissue being dark bluish green. The spaces of the cancellous tissue were shown to be filled with "gorgeously-coloured corpuscles, in ruby and yellow." Hairs and hair-follicles were green.

These experiments were made chiefly on sections of a kitten's tail. The directions for operating are: When the sections have become tolerably dark blue in appearance wash them rapidly in spirit of wine, dehydrate *rapidly* in absolute alcohol, and mount in Klein's dammar.

179. Picro-Carmine and Methyl-Green (*Max Flesch's method*[2]).—Sections of cartilage, skin, and glands made from tissues hardened in Müller's solution and alcohol, were stained with picro-carmine, and subsequently (not "previously," as erroneously stated in 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 883) with an aqueous solution of commercial methyl-green made of such a strength that the sections are just distinguishable in a watch-glassful of the solution when placed on a light ground.

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[1] 'Journ. Roy. Mic. Soc.' (N.S.), i (1881), p. 868.

[2] 'Zool. Anz.,' 123 (1882), p. 554.

The method is easy, gives good differentiations, but the stain does not appear likely to be permanent. Mount in balsam.

180. Picro-Carmine and Logwood (*Heneage Gibbes's method*[1]).—Gibbes found this combination answer better than any other stain for spermatozoa. It is also good for sections. He recommends that after staining with picro-carmine the sections should be placed for an hour in water acidulated with a few drops of acetic or picric acid; they take the second stain better, and do not fade afterwards.

181. Picro-Carmine, Rosein, and Anilin-Blue; or Picro-Carmine, Anilin-Violet, and Anilin-Blue; or Picro-Carmine, Anilin-Violet, and Iodine-Green; or Picro-Carmine, Rosein, and Iodine-Green (*Heneage Gibbes's method*[2]).—Make a dilute solution of picro-carmine (about 10 drops to a watch-glass of water), stain in it for about half an hour, wash out for an hour in water acidulated with a few drops of acetic or picric acid, and then double stain either with rosein and anilin blue (No. 194), or with anilin-violet and anilin-blue (No. 195), or with anilin-violet and anilin-green (No. 196), or with rosein and anilin-green (No. 197).

H. Gibbes says of these methods that their great utility consists in their power of differentiating glandular structures according to their secretions. In a section of a dog's tongue "the ordinary mucous glands will be found to have taken on a purple colour, while the serous glands which supply the secretions to the taste-organs stain a totally different colour. In an examination I lately made in a case of dysidrosis," he continues, "I was able to stain the duct of the sweat gland an entirely different colour from the surrounding tissues, and so demonstrate its relation to the vesicles."

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[1] 'Journn. Roy. Mic. Soc.,' iii (1880), p. 390.

[2] L. c., p. 392.

182. Picro-Carmine and Eosin (*Lang's formula*[1]).—Take 50 parts 1 per cent. picro-carmine, 50 parts 2 per cent. eosin (aqueous solution). The objects, previously hardened in alcohol, are left in the mixture half to four days. Wash out the picrin by 70 per cent. alcohol, which must be frequently changed, and be followed by 90 per cent. and absolute alcohol until no more eosin is dissolved out.

A nuclear stain. It is a double stain. The function of the eosin appears to be that by reason of its superior penetration it serves as a vehicle to carry the picro-carmine through tissues which would otherwise be impermeable to that substance.

For *Turbellaria.*

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[1] 'Journ. Roy. Mic. Soc.,' ii, 163. 'Zool. Anz.,' ii, p. 45. 'Mitth. d. Zool. Stat. zu Neapel,' Bd. ii, p. 1, *et seq.*

CHAPTER XVII.

OTHER MULTIPLE STAINS.

183. Methyl-Green and Eosin (*Calberla's formula*[1]).—Mix 1 part of eosin with 60 parts of methyl-green, and dissolve the mixture in warm 30 per cent. alcohol.

Sections stain in this solution in five or ten minutes; they should be quickly washed in successive alcohols, and mounted in balsam or glycerin.

In general nuclei of epithelia stain reddish violet or blue, nuclei of connective tissue green or greenish blue, the extra-nuclear parts of cells being rose-red. Striated muscles stain red, their nuclei green, smooth muscles green, and their inter-cellular substance red. The cells of ducts of salivary glands stain blue, the secreting-cells red, and the cells of the surrounding connective tissue green to greenish blue.

184. Eosin and Methyl-Green (*Moore's method*[2]).—A slide is prepared with a layer of blood dried on to it, and is then flooded with the following solution: Eosin 5 grains, water 4 drachms, alcohol 4 drachms (dissolve the eosin in the water and add the alcohol). After three minutes the stain is washed off with water, and the slide treated for two minutes with a solution of 5 grains of methylanilin-green to the ounce of water. Wash, dry, mount with balsam. Red corpuscles red, nuclei and leucocytes bluish green.

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[1] 'Morph. Jahrb.,' iii (1877), 3 Hft., p. 625.

[2] 'The Microscope,' ii (1882), p. 73. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 714.

185. Eosin and Anilin (*Schiefferdecker's methods*[1]).—Alcoholic eosin is prepared according to Fischer's formula (No. 158) ('Arch. Mik. Anat.,' xii, p. 349), and 1 per cent. aqueous solutions are prepared of dahlia, methyl-violet, and anilin-green. (It should be noted that anilin-green is *not* the same as either methyl-green or emerald-green.) The section is stained in a watch-glassful of alcohol to which a few drops of the eosin solution have been added, for from half an hour to several hours, rinsed in water, and transferred to a watch-glass containing one of the anilin solutions. In a few minutes it will appear stained almost black. The solution may be diluted and longer time taken if desired. Or, in order not to lose sight of a section in the dark fluid, it may be laid on a strip of blotting-paper which is immersed with it. Now rinse the section in water and pass it into alcohol. As soon as the two stains are sufficiently washed out the preparations are brought into oil of cloves. (If they are now seen to be not sufficiently washed out they may be returned to the alcohol.) The oil of cloves must be very completely removed from the preparations before mounting. Mount in chloroform-balsam.

Preparations may be hardened in alcohol or in the eosin tincture itself. Chromic acid may be used. The anilin-green stains green, the other two anilins blue; the blue colour appears principally in nuclei, the red of the eosin in the protoplasm. A very selective stain, most tissues taking on a special colouration, so matrix of cartilage blue, and the "cell-membrane" red; elastic fibres bright red, connective-tissue fibres dark rose; bone intense scarlet, blood scarlet. Very satisfactory for skin, bone, and cartilage, central nervous system, glands of the digestive system, liver, trachea, and larynx, lungs, bladder (of amphibia), testes, prostate, ovary, uterus, tubi, vaginæ, mammæ, lymphatic glands. Not useful for muscle, kidney, or peripheral nervous system, or sense-organs.

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[1] 'Arch. Mik. Anat.,' xv (1878), p. 30.

186. Hæmatoxylic Eosin (*Renaut's formula*[1]).—In order to cure the diffuseness of stain which is the capital defect of aqueous eosin solutions, Renaut combines eosin with hæmatoxylin.

It had already been proposed (by Wissotsky, of Kazan, in 1876) to double stain with hæmatoxylin and eosin in succession, but this method has many defects. Frequent washings are necessary, and moreover eosin solutions precipitate Boehmer's hæmatoxylin, throwing down granular or flaky deposits which obscure the preparations. Renaut, having remarked that this precipitation does not occur if the two solutions are mixed in the presence of neutral glycerin, proceeds as follows:

Make a mixture of equal volumes of neutral glycerin and saturated solution of eosin (in alcohol or in water, according as pure eosin or potassic eosin is employed). Add Boehmer's hæmatoxylin (No. 94), drop by drop, until the green fluorescence of the mixture becomes almost imperceptible. Filter, and you will obtain a violet-coloured solution of hæmatoxylic eosin.

To be used in the same way as picro-carmine.

Mount in saline glycerin (1 per cent.) or in balsam. (In the latter case both the alcohol used for dehydrating, and the oil of cloves used for clearing, should be charged with eosin).

Osmic and chromic-acid preparations stain selectively, and with precision, nuclei violet, connective tissue pearl-grey, elastic fibres and blood-corpuscles deep red, protoplasm and axis cylinders " intense light red."

The stain has a specific action on the cells of salivary and gastric glands. Sections of the salivary glands of *Helix pomatia*, demonstrate the existence of two kinds of gland-cells, the one sort staining bright rose, the other (of similar appearance whilst unstained) becoming of an intense blue (mucus-cells).

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[1] 'Comptes Rendus,' 1879, p. 1039 (1re sér.).

Similar reactions are obtained with the salivary glands of mammalia (and particularly with the sub-maxillaries of Solipedes). (Ass : fix in osmic acid of 1 per cent. for twenty-four hours, wash in water, harden for twenty-four hours in 90 per cent. alcohol, make sections.) The mucus-cells become pale blue, the salivary ferment-cells (crescent-cells of Gianuzzi) intense rose.

187. Logwood and Iodine-Green (*Stirling's method*[1]).—Stain *not too deeply* with logwood, and then stain with iodine-green. (Mucous glands of tongue green, serous glands hæmatoxylin.)

188. Eosin and Iodine-Green (*Stirling's method*[2]).—Stain in alcoholic solution of eosin, wash in acidulated water (acetic or hydrochloric acid, 1 per cent.), stain with iodine-green. (Developing bone and cerebellum.)

189. Eosin and Logwood (*Stirling's method*[3]).—For cerebrum. (General substance eosin tint, nerve cells lilac.)

190. Rose Bengale and Iodine-Green (*Griesbach's method*[4]).—The method consists in staining very quickly in a strong aqueous solution of rose bengale (the section must have been soaked in water before bringing into the stain), washing out with water, and staining for a few seconds in iodine-green. The sections may then be mounted, or may be further treated with bleu de Lyon. This is done by treating them for five minutes with absolute alcohol, and staining for two or three seconds in a solution of bleu de Lyon in 40 per cent. alcohol. The sections appear not to take the blue stain, but it becomes visible as soon as they are mounted. They are to be dehydrated in absolute alcohol, cleared in oil of anise-seed of 0·99 sp. gr., and 1·811 refractive index, and mounted in dammar. The method gives in some cases very striking differentiations, but the results are by no means constant, and do not admit of being generalised.

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[1] 'Journ. of Anat. and Physiol.,' xv (1881), p. 353.

[2] Ibid., p. 354.

[3] Ibid., p. 354.

[4] 'Zool. Anzeig,' 135 (1883), p. 172.

191. Gold Chloride and Anilin (for growing bone) (*Stirling's method*[1]).—*Tail of young rat.*—Remove the skin, divide into small pieces, which treat as follows: lemon juice, five minutes; wash water; gold chloride, 1 per cent., an hour or an hour and a half; wash; formic acid 25 per cent., twenty-four hours (in the dark). Decalcify with chromic and nitric acid. Keep in alcohol. Sections stained with (*e.g.*) alcoholic solution of rosein, followed by aqueous solution of iodine-green. Mount in dammar.

192. Gold Chloride and Saffranin (*Pfitzner's method*[2]).—Gold chloride 1 per cent., with a trace of HCl, fifteen to thirty minutes, in the dark; wash; reduce in daylight (twelve to twenty-four hours) in 5 per cent. formic acid; wash; and stain with saffranin. Mount in dammar. For epidermis of tadpoles.

193. Atlas Scarlet and Anilin-Blue (*Richardson's method*[3]).—Stain in a "deeply tinted watery solution of atlas scarlet, made by adding drop by drop to filtered water a very deeply coloured solution of the scarlet in Price's glycerin. To the watery solution a few drops of alcohol may be added. Examine the sections from time to time, say every third day," until they are found to be stained of a deep scarlet tint. Wash in methylated spirit. Stain for from fifteen to twenty minutes in a "blue watery solution, made by adding a drop or two of a deep-coloured solution of soluble blue in glycerin to filtered water." (If the blue fluid shows signs of fading after the sections have been in it for a few minutes, add a drop or two of glacial acetic acid; or the acid may be added to the blue fluid before bringing the sections into it.) Wash in methylated spirit and dehydrate in absolute alcohol. Clear with clove oil and mount in dammar.

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[1] 'Journ. of Anat. and Physiol.,' xv (1881), p. 354.

[2] 'Morph. Jahrb.,' vii (1882), p. 731. 'Journ. Roy. Mic. Soc.' N.S.), ii (1882), p. 883.

[3] 'Journ. Roy. Mic. Soc.' (N.S.), i (1881), p. 573.

For spinal cord: cells blue or bluish grey, with darker nuclei, axis cylinders of the same dark tint, white substance of Schwann scarlet.

The author does not recommend the process for tissues in general, merely stating that it is "capable of producing excellent didactic results."

194. Rosein and Anilin-Blue (*Heneage Gibbes's method*[1]).—Stain in a dilute solution of rosein in alcohol. Wash in methylated spirit. Bring the sections into dilute aqueous solution of anilin-blue: "the spirit causes the section to spread itself out and float on the watery solution, and it may be seen taking on the new colour." Wash in water, and bring into spirit, when, generally, more of the first colour will come out. When quite clean, mount in the usual way.

H. Gibbes says "this is a very good process for double staining, and if the section is of the same thickness throughout the staining will be perfectly even, and each colour will have picked out those tissues for which it has a special affinity."

195. Anilin-Violet and Anilin-Blue (*Heneage Gibbes's method*[2]).—Make an alcoholic solution of the violet, and an aqueous solution of the blue, and stain first with the violet and then with the blue, as directed for rosein and anilin-blue, *supra*, No. 194.

196. Anilin-Violet and Iodine-Green (*Heneage Gibbes's method*[3]).—Make an alcoholic solution of the violet, and an aqueous solution of the green, and stain first with the violet and then with the green, as directed for rosein and anilin-blue, *supra*, No. 194.

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[1] 'Journ. Roy. Mic. Soc.,' iii (1880), p. 391.

[2] L. c.

[3] L. c.

197. Rosein and Iodine-Green (*Heneage Gibbes's method*[1]).—Make an alcoholic solution of the rosein, and an aqueous solution of iodine-green, and stain first with the rosein, and then with the iodine-green, as directed for rosein and anilin-blue, *supra*, No. 194.

198. Gold-Chloride and Anilin (*Heneage Gibbes's method*[2]).—Stain with chloride of gold in the usual manner, and then with one of the four double stains just described.

199. Anilin Double-Stains for Blood (*V. Harris's experiments*[3]).—A lengthy series of experiments on combinations of anilins. I shall abstract them as briefly as possible, because they do not appear likely to be of much use in general histology. Only one tissue—blood—was experimented on; and the conditions under which the dyes were employed were such as can in nowise be employed in general histological research. Layers of blood were dried, treated with aqueous or dilute solution of one of the dyes, washed with water, dried in the flame of a spirit-lamp, treated with aqueous or dilute solution of the second dye, washed with water, dried, and mounted in Canada balsam.

Eosin and aurin.—Unsuccessful, the solution of aurin having to be made with absolute alcohol, and entirely driving out the eosin.

Fuchsin and anilin-primrose.—Nuclei, yellowish crimson, remainder of the coloured corpuscles light yellow. Not a good combination.

Rosein and iodine-green.—Coloured corpuscles bright red, with bluish-green nuclei. An excellent combination.

Fuchsin and methylen-blue.—Coloured corpuscles pink, or green with a pink edge, nuclei deep blue. One of the most successful combinations.

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[1] L. c.

[2] L. c.

[3] 'Quart. Journ. Mic. Sci.' (N.S.), xc (1883), p. 292.

Fuchsin and soluble blue.—Stroma light blue, nuclei red. As good a combination as the last.

Eosin and methyl-violet.—Unsuccessful; did not give a double-stain.

Fuchsin and Bismarck brown.—Stroma a fine brown, nuclei red.

Eosin and vesuvin.—Stroma light brown, nuclei deep pink. A very successful combination.

Iodine-green and Bismarck brown.—Stroma brown, nuclei green.

Iodine-green and flamingo.—Stroma pink, nuclei deep bluish green.

Malachite-green and ponceau.—Stroma pink, nuclei green. (The green not permanent.)

Malachite-green and fluorescin.—Stroma yellow, nuclei yellowish green. (The green not permanent.)

Malachite-green and aurin.—Entirely unsuscessful.

Iodine-green and anilin-primrose.—Stroma yellow, nuclei green.

Iodine-green and bleu de Lyon.—Quite unsuccessful.

Malachite-green and methyl-violet.—Stroma pinkish yellow, nuclei light purple. Not good.

Hoffman's violet and Bismarck brown.—Stroma light brown, nuclei reddish brown. An excellent combination.

Hoffman's violet and flamingo.—Nuclei and stroma two shades of mauve.

Gentian-violet and anilin-scarlet.—Unsuccessful.

Gentian-violet and eosin.—Stroma light pink, nuclei deep red.

Hoffman's violet and tropæolin.—Entirely failed.

Gentian-violet and anilin-primrose.—Stained two shades of *green.*

Methyl-violet and methylen-blue.—Stroma pink, nuclei blue. One of the best combinations.

The only entirely successful combinations were:—rosein +

anilin-green; fuchsin + methylen-blue; fuchsin + Bismarck brown; eosin + vesuvin; iodine-green + Bismarck brown; Hoffman's violet + Bismarck brown; anilin-violet + methylen-blue.

Harris thinks he has proved that neither iodine-green nor malachite-green are permanent. He remarked that in all cases it was necessary that *the solutions should be quite fresh.*

CHAPTER XVIII.

HARDENING AGENTS.

200. If this chapter had been written ten years ago, it would have had a far greater importance than can now be claimed for it, and I should have considered it necessary to treat it with far greater detail than now seems desirable. The reason of this is that methods of imbedding have now been brought to such a degree of perfection that the thorough hardening of soft tissues that was formerly necessary in order to cut thin sections from them is now, in the majority of cases, no longer necessary; by careful infiltration with paraffin or some other good infiltration mass, most soft objects can be satisfactorily cut with no greater an amount of previous hardening than is furnished by the usual passing of the tissues after fixing through successive alcohols in order to prepare them for the paraffin-bath. Almost the only exceptions to this statement are, I believe, to be found in the cases in which it is desired to cut very large sections, such as sections of the entire human brain. Such an organ as this cannot be duly infiltrated with alcohol in a few hours, and it is doubtful whether it can be duly infiltrated with paraffin or any other imbedding mass in any reasonable time. The processes employed for hardening such specimens as these will be described when treating of the organs in question; in this chapter I confine myself to such general statements concerning the employment of the usual hardening agents as appear likely to be generally useful.

Hardening agents may conveniently be divided, from a practical point of view, into two groups, according to the nature of the modifications they produce in tissues. Those of the first group, which consists of alcohol, nitric acid, and picric acid, appear to act mainly by coagulating the albumen of the tissues, without in any way entering into chemical combination with their components; this group possesses the great advantage of in nowise interfering with subsequent staining (nitric acid and picric acid being readily removed from the tissues by means of alcohol). The second group consists of osmic acid, of chromic acid and its salts, and of palladium chloride. These appear in some way to chemically combine with some of the components of the tissues, or to produce inorganic precipitates in their substance (Mayer). These combinations or precipitates cannot be totally removed from the tissues by washing, or by any method except bleaching by chlorine (which causes the tissues to become as soft and yielding as they were before hardening). This group is therefore unfavorable to subsequent staining. The hardness imparted by the reagents of this group is much more favorable for cutting than that produced by those of the first group. Corrosive sublimate appears to belong to this group on account of the nature of the reaction, but it does not interfere with subsequent staining, and sometimes even forwards it.

201. Alcohol.—Alcohol should always be taken strong, *i.e.* not less than 95 per cent.; absolute alcohol is sometimes necessary. Large quantities of alcohol should be taken. The alcohol should be frequently changed, or the tissue should be suspended near the top of the alcohol, in order to have the tissue constantly surrounded with pure spirit (the water and colloid matters extracted from the tissue falling to the bottom of the vessel). Many weeks may be necessary for hardening large specimens.

202. Nitric Acid.—Nitric acid is taken of a strength of from 3 per cent. to 10 per cent. or more, and may be allowed to act for two or three weeks. It gives, thus employed (10 per cent. to 12 per cent.), very tough preparations of brain. It is also conveniently used by employing a very short immersion and completing the hardening with alcohol, in which case it is properly considered as a fixing agent. See the information given under this head, No. 28.

203. Picric Acid.—Picric acid should always be used in saturated solution, and very large quantities should be taken. The reaction need not be prolonged more than a few hours. In any case, the degree of hardness obtained by the use of picric acid alone is so small that this reagent is more properly considered to be merely a fixing agent. The hardening is, however, quite sufficient to afford sections of most objects after dehydrating and imbedding in an infiltration mass.

204. Corrosive Sublimate.—Sufficient directions for the employment of this reagent are given in the chapter on FIXING AGENTS, No. 29.

205. Osmic Acid.—Osmic acid is much more useful as a fixing agent than as a hardening agent. Long immersion in osmic acid is sure to cause blackening, and may cause brittleness in the tissues. The strengths employed for hardening vary from $\frac{1}{5}$th per cent. to 1 per cent. and the tissues are left in the solutions for twelve to twenty-four hours, seldom more. See the further information as to the employment of this reagent given in the Chapter on FIXING AGENTS, No. 12.

206. Chromic Acid.—Chromic acid is generally employed in strengths of $\frac{1}{5}$th per cent. to $\frac{1}{2}$ per cent., the immersion lasting a few days or a few weeks, according to the size and nature of the object. Mucous membrane, for instance, will harden satisfactorily in a few days, brain will require some six weeks.

Large quantities of the solution must be taken (at least 200 grammes for a piece of tissue of 1 centimetre cube, Ranvier). The solution should be taken weak at first, and the strength increased after a time. The objects should be removed from the solution as soon as they have acquired the desired consistency, as if left too long they will become brittle. They may be preserved till wanted in alcohol (95 per cent.). It is well to wash them out in water for twenty-four or forty-eight hours before putting them into the alcohol. I think it is frequently useful to add a little glycerin to the hardening solution, there is less brittleness, and I think less shrinkage.

Further directions for the employment of chromic acid will be given in the special paragraphs. Chromic acid is a most powerful and rapid hardening agent; it has the defect of a great tendency to cause brittleness.

207. Chromic and Osmic Acid (for auditory organs) (*Max Flesch's formula*[1]) :

Osmium	0·10 parts.
Chromic acid . . .	0·25 ,,
Distilled water . .	100·00 ,,

Specimens are to be placed fresh in this mixture, and may be left there from twenty-four to thirty-six hours, without risk of the osmic acid overstaining them. To complete the decalcification, ¼ to ½ per cent. chromic acid is then employed (for small specimens, temporal bone of young mouse, for instance, this is not necessary). Dehydration in alcohol then follows. The organs may then be at once cut and mounted in glycerin, or they may pass through absolute alcohol, turpentine, and solution of paraffin in turpentine, into an imbedding mass of paraffin and tallow, then be cut, cleared with turpentine, and mounted in balsam.

The cilia of the hair-cells are mostly lost by this process, but the varicose nerve-fibrils of the organ of Corti are well preserved. The mixture causes greater shrinking than osmium alone. The author recommends it for preparations of growing epiphyses, for glands, retina, conjunctiva, cornea, eyelids.

208. Chromic Acid and Platinum Chloride (*Merkel's solu-*

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[1] 'Arch. Mik. Anat.,' xvi (1878), p. 300.

tion[1]).—Equal parts of 1·400 solution of platinum chloride, and 1·400 solution of chromic acid. "Prof. Merkel states that he allowed from three to four days for the action of the fluid" for the retina; for annelids Eisig employs an immersion of three to five hours, and transfers to 70 per cent. alcohol; for small leeches Whitman finds "one hour sufficient and transfers to 50 per cent. alcohol."

209. Bichromate of Ammonia. 2 to 5 per cent. Several weeks will be necessary in most cases. This and the other chromic salts take five or ten times as long as chromic acid to do their work, but the hardening is more perfect, the preparations never becoming brittle. The ammonium salt has come of late to be generally preferred to the potassium salt.

210. Bichromate of Potash.—Same strength as bichromate of ammonia.

211. Chromate of Ammonia. 5 per cent. For twenty-four hours (intestine, Klein).

212. Müller's Solution.

Bichromate of potash	2—2½ parts.
Sulphate of soda	1 „
Water	100 „

The duration of the reaction is about the same as with the simple solutions of chromic salts.

213. Potassium Bichromate and Cupric Sulphate.—A variation of Müller's solution; instead of 1 per cent. sulphate of soda you take ½ per cent. sulphate of copper. The hardening properties are superior to those of Müller's solution. This formula is, I believe, now very generally employed in Germany. I do not know by whom it was suggested.

214. Chromic Acid and Alcohol.—Chromic acid of the strength given above diluted with half its volume of 95 per cent. alcohol. (*Solutions* of chromic acid should always be taken for mixture with alcohol, as if chromic acid be dissolved directly in alcohol a very violent reaction takes place.)

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[1] Merkel, 'Ueber die macula lutea, &c.,' 1870, p. 19. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 871.

215. Palladium Chloride (*F. E. Schulze's method*[1]).—Single palladium chloride (Pd Cl) may be prepared by dissolving palladium-ore in hydrochloric acid containing a certain quantity of nitric acid, and evaporating to dryness. To dissolve the chloride water acidulated with HCl must be employed; to dissolve the chloride of commerce take for 10 grammes of the chloride 1 litre of water and 4 to 6 drops HCl. The solution will be complete in twenty-four hours. Such a solution may be conveniently kept in stock and diluted as required for use. The 1·100 solution should be dark red-brown, a 1·800 solution pale yellow, like a 0·2 per cent. chromic-acid solution.

The free acid of the solution is necessary to the reaction it is intended to produce in the tissues.

To harden tissues containing much connective tissue, as, for example, the ciliary body, a 1·800 solution should be taken. A piece of the tissue of the size of a bean may be placed in 30 c.c. of the solution, care being taken to remove all structures that would be an obstacle to the penetration of the chloride, which, for many tissues, has far less penetrating power than chromic acid. This is particularly the case with structures that are poor in connective tissue, as certain nerves, brain, and spinal cord, and deeply-stratified epithelia, such as human epidermis. Very small pieces, therefore, of such tissues must be employed; of nerves such may be chosen as are rich in connective tissue, as the anterior part of the *N. opticus*, for the solution has great penetrating power in regard to all connective tissue.

Hardening is generally complete at the normal temperature in two or three days, but the specimens may remain in the solution for weeks or months without hurt. The consistency of the hardened tissues is far more favorable for cutting than that obtained by chromic acid or Müller's solution, whilst the fine details of structure are equally well preserved. Certain of the tissue elements are found to be stained yellow, brown, or black, whilst others remain colourless. Protoplasm in general appears dark yellow, striated muscle brownish yellow, smooth muscle straw yellow, medullated nerve ink-black. Hyaline membranes and elastic fibres remain transparent and are only very slightly tinted with yellow. The gelatin-yielding interstitial substance of connective tissue remains perfectly colourless, so that muscle fibres and cells can be at once distinguished from their connective-tissue matrix; further, this unstained matrix will stain deeply with carmine and other stains, whilst the structures coloured by the chloride refuse to stain in the least. Sections must be well freed from the chloride before mounting in glycerin or they will blacken with time.

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[1] 'Arch. Mik. Anat.,' iii (1867), p. 477.

CHAPTER XIX.

THEORY OF IMBEDDING, WITH A WORD ON MICROTOMES.

216. Imbedding methods may conveniently be divided into two classes, distinguished by the end it is intended to compass by their employment. In the one it is merely proposed so to surround an object, too small or too delicate to be firmly held by the fingers or by any instrument, with some plastic substance that will support it on all sides with firmness but without injurious pressure, so that by cutting sections through the composite body thus formed, the included object may be cut into sufficiently thin slices without distortion. This is *simple* imbedding. Its object is very easily attained in a variety of ways of which the simple process of immersing the object to be cut in a molten mass of some such material as wax, which when cold acquires a fit consistency for the cutting of thin slices, may be taken as a type. A further object is proposed in the case of the other class of methods, which may be designated methods of *interstitial* imbedding or *infiltration* methods. In these it is proposed to fill out with the imbedding mass the natural cavities of the object in order that their lining membranes or other structures contained in them may be duly cut *in situ*, or, going a step further, it is proposed to surround with the supporting mass not only each individual organ or part of any organ that may be present in the interior of the object, but each separate cell or other anatomical element, thus giving to the tissues a consistency they could not otherwise possess, and ensuring that in the thin slices cut from the mass all the details of structure will precisely retain their natural relations of position. Such a process of imbedding is at the same time practically a process of hardening in so far as it enables us to give to tissues a degree of firmness that could otherwise only be obtained by the employment of chemical processes such as prolonged treatment with chromic acid and the like.

The principle of the methods of this second class is either, like that of the first, that by immersion of the object to be cut in some material that is liquid while warm and solid when cold, all the parts of the object may be duly surrounded by the supporting mass (the second class differing from the first chiefly in the employment of materials possessing greater power of penetration whilst liquid, in longer immersion in the liquid mass, and in such previous preparation of the object, by soaking in some liquid that is a solvent of the imbedding material, as makes it more readily susceptible of infiltration by the latter); or the processes may be based on another principle, namely that of the employment of substances which whilst in solution are sufficiently fluid to penetrate the object to be imbedded, whilst at the same time after the evaporation or removal by other means of their solvent, they acquire and impart to the imbedded object sufficient firmness for the purpose of cutting. The collodion process sufficiently exemplifies this principle. If a piece of soft tissue be dehydrated, and soaked first in ether and then in collodion, and if the ether contained in the collodion be allowed slowly to evaporate, the tissue and surrounding mass of collodion will acquire a consistency such as to admit of thin sections being cut from them.

The egg-emulsion process, in which a mass that is liquid whilst cold is coagulated by heat, forms a class by itself.

In any of these cases the material used for imbedding is technically termed an "imbedding-mass." (*Einbettungsmasse*:—*masse d'inclusion.* Imbedding methods are spoken of by French writers as *méthodes d'inclusion*, or *méthodes d'enrobage*).

217. Before setting out the formulæ for the different imbedding masses that have been recommended, it is necessary to say a few words as to the details of manipulation in the process of imbedding.

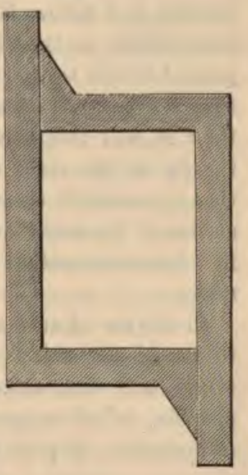
To imbed an object in such a substance as liver or spinal cord (which does not strictly come under the category of an imbedding mass at all, as defined above) nothing more is necessary than to take a piece of fresh liver or cord of convenient dimensions, scoop-out in it a hole of the size of the object to be imbedded, place the object in the hole, and immerse the mass in alcohol until such time as the mass is sufficiently shrunken and hardened to hold the object firmly and permit of section cutting.

If pith be employed, a cylinder of pith is halved longitudinally, a cavity corresponding to the object to be imbedded is made by scooping out the inner face of either half-cylinder, the object placed in position between these, and the cylinder pushed into the well of a microtome (which it should fit accurately), and moistened with alcohol (or other suitable liquid) in order that the pith may swell round the object. It should be noted that *it is better to make the cavity in the pith by simple pressure and kneading* (*e.g.* with the handle of a scalpel) than *by excavation of material;* the pith-cells that have been flattened and pushed to one side by the kneading, tend to regain their normal form and position during the soaking in alcohol, and their resilience causes the imbedded object to be grasped with an often surprising tightness. If the cylinder of pith does not fit the holder of the microtome accurately in the dry state (which it should do if possible) it should be wedged in by means of strips or thin wedges of *kneaded* pith inserted dry, and the whole afterwards soaked. With well-hardened objects this method, if skilfully carried out, allows of very accurate section cutting.

218. *Simple* imbedding in a melted mass such as paraffin is performed in one of the following ways. A little tray or box or thimble is made out of paper, some melted mass is poured into it, at the moment when the mass has cooled so far as to have a consistency that will not allow the object to sink to the bottom, the object is placed on its surface, and more melted mass poured on until the object is enclosed. Or the paper tray being placed on cork, the object may be fixed in position in it whilst empty by means of pins and the tray filled with melted mass at one pour. The pins are removed when the mass is cold.

In either case when the mass is cold, the paper is removed from it before cutting.

For small objects it is often convenient to employ, instead of paper trays, small square porcelain moulds (such as moist water-colours are sold in). The mass when cold is scooped out of the mould with a spatula.



"In Professor Leuckart's laboratory are used boxes made of two pieces of type-metal. Each of these pieces has the form of a carpenter's "square" with the end of the shorter arm triangularly enlarged outwards. The box is constructed by placing the two pieces together on a plate of glass which has been wetted with glycerin and gently warmed. The area of the box will evidently vary according to the position given to the pieces, but the height can be varied only by using different sets of pieces. In such a box the paraffin may be kept in a liquid state by warming now and then over a spirit-lamp, and small objects be placed in any desired position under the microscope."[1]

In the case of the employment of a cylinder-microtome (Ranvier's, Rutherford's) it has been recommended that the object be simply placed in the well of the microtome and imbedded by pouring melted mass on to it, holding it in position meanwhile if need be by means of forceps. This is the simplest of all methods, and may be used in cases in which accuracy is not desired. But it is impossible to ensure exact results by this means. It will be found that unless special precautions are taken the molten mass will shrink away from the sides of the well on cooling and the cast will lie more or less loosely in the well. Then when sections are made it will frequently be found that the cast has a tendency to turn round, and a tendency to rise in the tube, and further progress will be impossible until the object is re-imbedded.

Shrinking of the mass away from the well may be more or less prevented by using masses of a certain composition (*e.g.* paraffin and tallow, No. 229), and by having the well made removeable, so that it may be warmed before the mass is poured into it to the same temperature as the mass itself, so that it may contract together with the mass on cooling; but then it will frequently be found that the mass clings so tightly to the sides of the tube as to put great strain on the micrometric screw, and submits to be pressed by the action of the screw instead of being duly raised *en masse* by it. Accurate section cutting under such conditions is impossible.

In the use of such a microtome, then, it is always advisable to imbed the object separately by one of the methods given above, and then either to cement it by means of a drop of paraffin, collodion, gum, or other suitable substance, to a cork or cylinder of pith that has previously been accurately adjusted to the bore of the well; or to pack the cast in the well by means of wedges of kneaded pith in the way explained above.

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[1] 'Journ. Roy. Mic. Soc.' (N.S.), ii, p. 880.

In the use of a microtome in which the object is held in jaws, as in the Rivet-Leyser or Thoma form, the object is imbedded in a cast of suitable shape by one of the above methods, and the cast is inserted directly into the jaws, or cemented to a piece of cork which is held by them.

219. Instructions for the preparation of objects, cutting, and further treatment of sections are given in the sections devoted to each mass. A word may, however, here be said as to a somewhat important detail of procedure, viz. the freeing of the sections from the imbedding-mass. In the case of imbedding by infiltration the mass must be removed from the tissues by means of an appropriate solvent, unless it be of such a nature as to become sufficiently transparent in the medium employed for mounting; and in the case of simple imbedding the same practice is very generally adopted. In this case, however, it is frequently better to prepare the object before imbedding it by covering it with a film of some substance that prevents the immediate contact of the imbedding-mass with the object and can itself be easily removed; for instance, if a tissue has been hardened in chromic acid and is to be imbedded in wax and oil, it should (after soaking in alcohol and wiping dry at the surface with blotting-paper) be rolled for a moment in gum-arabic solution, dried for a few seconds, and then imbedded. The sections when cut are floated into water which dissolves the layer of gum, and the sections are obtained free from the wax without any trouble. Or collodion may be used instead of gum; it will dissolve out naturally in the oil of cloves used for clearing, and the cast of wax fall away from the sections.

220. The following general statements may perhaps serve to guide the student in the choice of an imbedding-mass for any particular purpose.

Paraffin (No. 231) used pure, as an interstitial mass, allows of the cutting of the thinnest sections.

Collodion (made with celloidin) does not afford sections quite so thin as those that can be obtained by means of paraffin; the collodion method is the least hurtful of all methods to delicate tissues; it is the surest and the easiest to carry out of all methods of interstitial imbedding.

Wax and oil is a trustworthy and very convenient simple imbedding-mass.

221. Section-Stretching.—The media with which the knife used for section-cutting should be moistened are indicated for each mass. Some masses are now generally cut *dry*, paraffin, for instance. By this means thinner sections are obtained, but a difficulty generally arises owing to the tendency of sections so cut to curl-up on the blade of the knife. It is often impossible by any means to unroll a thin section that has curled. To obviate this, during the cutting the edge of the section that begins to curl is caught and held down on the blade of the knife by means of a small camel-hair brush with a flat point, or with a small spatula made by running a piece of paper on to the back of a scalpel, or by means of an ingenious little instrument called a "section-stretcher." This consists essentially of a little metallic roller suspended over the object to be cut in such a way as to rest on its free surface with a pressure that can be delicately regulated so as to be sufficient to keep the section flat without in any way hindering the knife from gliding beneath it.[1]

Vaselin added to paraffin gives a mass that reduces the tendency to curl on the part of the sections. A curled section may sometimes be caused to unroll by putting it into alcohol or by gently warming it. A delicate sawing movement given to the section-knife will sometimes suffice to prevent curling.

I have sometimes found it convenient, instead of holding the section down by the edge that begins to curl, to run under it (*i.e.* between the edge of the section and the blade of the knife) a tiny drop of alcohol. The capillary attraction of the fluid is sometimes sufficient to hold the section down to the knife. As to melting-points of paraffin, see below, 223.

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[1] See the descriptions of various forms in 'Zool. Anzeig,' vol. vi (1883), p. 100; and in 'Journ. Roy. Mic. Soc. (N.S.), vol. iii, pp. 450 and 916.

222. It may here be noted that objects imbedded by infiltration with paraffin may be preserved indefinitely in the dry state till required for cutting, the paraffin constituting a sufficient preservative medium, and it is not necessary to keep the blocks in alcohol. Objects imbedded in collodion (or celloidin) may be kept till wanted in spirit (36°).

222 a. A Word on Microtomes.—It is no part of the purpose of this work to discuss instruments, yet a word on this subject may be very helpful to the student. The freezing microtome so generally employed in England is less than any other form adapted to the wants of the *zoologist.* Very thin sections can be obtained by it more readily than with any other microtome, but they are of little use when obtained. The relations of the parts of the organs are deranged by the freezing and by the thawing, and the aqueous nature of the process prevents it from being readily applicable to the mounting of *series* of sections. The microtome of the *zoologist,* therefore (I am not writing for pathologists or for dilettanti), must be an *imbedding* microtome. The two most important points to be attended to in the choice of such a microtome are the *object-holder* and the *knife motion.* The object-holder should *never,* as is usual in the English forms, be a well in which *the imbedded object* is raised by a screw; the principle of construction should always be that the *object-holder be raised* in its entirety by the screw, *not* the object alone. The *knife motion* should always be mechanical; cutting of sections with a free knife held in the hand is a primitive process, by which only coarse sections can be obtained, and that at the expense of much time and attention. Remember that the mean thickness to which sections are now cut is 5μ, half a hundredth of a millimetre, or less than $\frac{1}{5000}$th of an inch. Clearly the free hand is not capable of producing *regular* series of sections of such a degree of fineness. The student should therefore be careful to provide himself with an instrument in which the knife is guided by a mechanism giving the required precision of stroke. Cylinder-microtomes with free knife motion should be unhesitatingly condemned by him.

Amongst microtomes fulfilling the conditions I have laid down various forms will be found almost equally convenient. Zeiss makes a good one, Schanz of Leipzig makes a good one, Reichert of Vienna makes a good one. All these are relatively cheap, and being at the same time perfectly efficient may be recommended; but I must say that to my mind the finest instrument of all is the Thoma sliding-microtome. If the student will obtain from R. Jung, Mechaniker in Heidelberg, a Thoma microtome, *medium size* (No. 2*a* or 4), with the newest Naples object-holder and newest form of knife and knife-holder, he will, in my opinion, be possessed of the *nec plus ultra* of microtomes.

This instrument is described in 'Journ. Roy. Mic. Soc.' (N.S.), vol. iii, p. 298; the new Naples object-holder (which I consider *essential* for the zoologist), is described and figured p. 915.

223. Paraffin-Imbedding.—Success in paraffin-imbedding depends in great measure on the choice of a suitable paraffin. It is recommended by Gaule to employ a bluish-transparent sort, which rings slightly when struck. Paraffin should be chosen *of a melting point suitable to the temperature of the laboratory.* Bourne recommends[1] 100° or 115°. Kossman[2] finds that for a temperature of 18° C. a paraffin of 48° C. melting point should be used, whilst "on hot summer days" the hardest kinds of paraffin must be used pure. He recommends that two kinds of paraffin, viz. of 56° and 36° melting point, should be kept in stock; intermediate sorts can be made by the melting together of these.

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[1] 'Quart. Journ. Mic. Sci.,' lxxxvii (1882), p. 335.

[2] 'Zool. Anz.,' 129 (1883), p. 20.

Chloroform appears to be in general the best solvent for preparing objects for the paraffin-bath. Bourne notes that "the paraffin must be melted in a small covered vessel in a water-oven, *great care being taken to keep it in a dry atmosphere.* The objects should be kept in the bath of melted paraffin *for some hours* before imbedding in the paper trays or otherwise." Bourne here evidently has *large* objects in view. Sufficiently small ones may be infiltrated in a few minutes.

In order to transfer the objects from the chloroform-bath to the paraffin-bath, choice may be made of two methods; either, as in Giesbrecht's method, the chloroform containing the objects is heated to the melting point of the paraffin, and the paraffin gradually added, and the mass kept at the melting point of the pure paraffin until all the chloroform is driven off; or, as in Bütschli's method, the objects are simply passed direct from chloroform into a solution of paraffin in chloroform, in which they remain until thoroughly impregnated (half to one hour), and which is then evaporated at the melting point of the paraffin. Bütschli recommends a paraffin solution melting at 35°. (Such a solution is made of about equal parts of chloroform and paraffin of 50° melting point.) Or, in the case of larger objects, instead of evaporating the chloroform (which is often a very long process, as the chloroform must be *completely* driven off, or the mass will remain too soft for cutting), Bütschli simply transfers them from the bath of paraffin solution to a bath of pure paraffin.

At *Naples* objects are prepared for the paraffin-bath, either in kreasote, oil of cloves, or chloroform. They are brought from this fluid into a bath of soft paraffin, heated to about 50° and kept there for one hour. They are then brought for half an hour into a bath of a mixture of hard and soft paraffin, kept also at about 50° C. (A mixture of 2 parts hard paraffin to 1 of soft is found to work well for the winter temperature of the laboratory at Naples.) They are then imbedded in paper trays, or in the type-metal boxes described above.

224. Kingsley's Imbedding Method.[1]—For small objects the following procedure may be found useful. The object is removed from the paraffin solution, the superfluous fluid is removed by means of blotting-paper, and the object placed on a cylinder of paraffin (or paraffin and vaselin). A piece of stout iron wire is now heated in the flame of a spirit-lamp, and with it a hole is melted in the end of the cylinder, the specimen is pushed into the melted paraffin and placed in any desired position. The advantages of the method lie in the quickness with which it can be performed, and in the fact that by the melting of so small a quantity of paraffin all risk of injury to the tissues by overheating is done away with.

This method may also be used for simple imbedding in the case of solid objects without cavities or irregular outline. They are transferred direct from alcohol to the paraffin-cylinder, and when sections are cut they readily separate from the shaving of paraffin without the application of turpentine.

225. Solvents of Paraffin.—The following solvents have been recommended for freeing sections from the paraffin with which they are infiltrated. Turpentine, warm turpentine, a mixture of 4 parts of essence of turpentine with 1 of kreasote, kreasote, a mixture of turpentine and oil of cloves, benzin, xylol, thin solution of Canada balsam in xylol (only applicable to very thin sections), hot absolute alcohol, naphtha, or any other paraffin oil of low boiling point. Any of these may be used, but naphtha and xylol are probably in most respects the best.

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[1] 'Amer. Mon. Mic. Journ.' iv (1883), p. 58, from "Scientific and Literary Gossip;" 'Journ. Roy. Mic. Soc.' (N.S.), 1883, iii, p. 444.

Amongst the solvents used for preparing objects for the paraffin-bath, the following are most to be recommended: chloroform, turpentine, oil of cloves, oil of bergamot.

CHAPTER XX.

FUSION IMBEDDING-MASSES.

226. Wax and Oil (*Stricker's formula, Roy's formula*[1]).—Wax and oil in equal parts. The proportions may be varied according as it is desired to obtain a harder or a softer mass. The above are the proportions recommended by other capable observers who have worked with this mass. The present writer finds it to present a good consistency. In cold weather somewhat more oil should be taken, and in very warm weather somewhat more wax.

The objects are prepared by dehydrating in alcohol, and soaking in oil of cloves until cleared. Wet the section-knife with turpentine on its upper surface. The sections are freed from the mass, if necessary, by means of turpentine (but *see ante*, No. 219).

227. Wax and Oil (*Foster and Balfour's formula*[2]).—Three parts of white wax to 1 of olive oil. (For the embryology of the fowl.) I have not tried it. Cut with a knife wetted with alcohol, kreasote, or clove oil.

Wax and oil form an excellent and trustworthy mass for simple imbedding.

28. Paraffin and Axunge (*Huxley and Martin's formula, Foster and Balfour's formula*[3]).—Five parts of paraffin melted with one part of paraffin oil and 1 of axunge. (For embryos of the fowl.) The embryo is dehydrated in alcohol and saturated with kreasote before imbedding. A good procedure for simple imbedding. The section-knife should be wetted with alcohol, kreasote, or clove oil.

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[1] 'Handbuch d. Geweblehre,' pp. xxiii and 1202.

[2] 'Éléments d'Embryologie,' p. 296 (1877).

[3] Ibid.

229. Paraffin and Tallow (*Seiler's formula*[1]).—Pure paraffin 2 parts, rendered mutton tallow 1 part. "If poured into the well of the microtome at a temperature of about 120°, it will not shrink away when cooling either from the tissue or from the wall of the well."

230. Paraffin with Turpentine (*Gaule's method*[2])—Dehydrate the object in absolute alcohol, and then saturate with oil of cloves. Put into turpentine for half an hour (or more for a large object), and then for an hour or more into a mix- of turpentine and paraffin kept melted on a water-bath at about 40° C. Remove for one hour to a bath of pure paraffin melted at a temperature of 60° C. Imbed in a paper tray.

The paraffin is removed from the sections if thick by means of a bath of turpentine, if thin by benzin dropped on to them on the slide. For exceedingly delicate specimens another method may be used. Lay the section on the slide, wet with absolute alcohol, and let the alcohol completely evaporate, leaving the specimen attached to the slide; carefully heat until the paraffin is softened or slightly melted. When cool let a few drops of benzin—best applied with a brush—run over the section until most of the paraffin is gone. When dry apply the cover-glass, and put a thin solution of Canada balsam in xylol to its edge. The xylol may be used instead of benzin, but it is more expensive. (It gives better results —AUTHOR.)

"Select paraffin, if possible, which is bluish-transparent, and which rings slightly when struck. The white opaque sort is by no means as good. Any addition of paraffin oil, turpentine, &c., to soften the paraffin, renders it granular and brittle, and is decidedly injurious to its cutting qualities."

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[1] 'Compendium of Microscopical Technology,' 1881, pp. 47, 48. 'Journ. Roy. Mic. Soc.' (N.S.), i, p. 840.

[2] 'Amer. Mon. Micr. Journ.,' iii (1882), p. 14. 'Journ. Roy. Mic. Soc.' (N.S.), ii, p. 428.

231. Paraffin and Chloroform (*Giesbrecht's method*[1]).—Chloroform is the readiest solvent of paraffin, and is at the same time the most easily evaporated. These properties render possible the following procedure. Objects to be imbedded are saturated with absolute alcohol, and then brought into chloroform (to which a little sulphuric ether has been added if necessary, in order to prevent the objects from floating). As soon as the objects are saturated with the chloroform, the chloroform and objects are gradually warmed up to the melting point of the paraffin employed, and during the warming small pieces of paraffin are by degrees added to the chloroform. So soon as it is seen that no more bubbles are given off from the objects, the addition of paraffin may cease, for that is a sign that the paraffin has entirely displaced the chloroform in the objects. This displacement having been a *gradual* one, the risk of shrinkage of the tissues is reduced to a minimum. (The paraffin mass should be kept for some time at its melting point over a water-bath, or better, in a dry oven, in order that the chloroform may be completely driven off, as the presence of chloroform makes the paraffin too soft for thin section cutting.)

The discovery of chloroform as a solvent for paraffin is, I believe, due to Bütschli and Blochman. Undoubtedly the procedure above described is one of the most exact methods of interstitial imbedding yet made known.

Sections are cut *dry*; if they have a tendency to roll they must be held down on the section-knife with a small, flat, camel's-hair brush or other suitable instrument.

232. Paraffin and Chloroform (*Bütschli's formula*[2]).—A solution of paraffin (? melting point) in chloroform saturated at ca 35°. For the manner of employing it, *see ante,* No. 223.

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[1] 'Zool. Anz.,' No. 4 (1881), p. 484.

[2] 'Biol. Central,' i (1881), pp. 591-2. 'Journ. Roy. Mic. Soc.' (N.S.), vol. ii, p. 708.

233. Paraffin-Imbedding (*Kossmann's method*[1]).—Kossmann prepares his objects in chloroform, and brings them *direct* from the chloroform into a bath of *pure* paraffin. He keeps two sorts of paraffin, viz. of 56° and 36° melting point, and mixes them in different proportions in order to obtain a mass of the desired consistence, which should vary according to the temperature of the workroom. He recommends for a temperature of 18° a mass of 48° melting point, and for hot summer days the hardest kinds of paraffin unmixed. He leaves his objects in the bath for from a few hours to two or three days. He uses an air-bath, with a Bunsen burner connected with a Kemp-Bunsen gas regulator; he keeps it constantly heated to 50°. He imbeds in tin-foil trays.

234. Paraffin + Ceresin + Vaselin Imbedding-mass (*Schulgin's formula*[2]).—Take paraffin of 55° melting point, and add ceresin quant. suff. If the mass thus obtained be too hard, add vaselin quant. suff. Ceresin resembles wax, but is stronger and tougher. Sections cut from the above mass and laid dry on the slide are not brittle. To prevent them curling during cutting they may be held down with a small brush.

235. Paraffin + Vaselin (*Frenzel's formula*[3]).—Paraffin 4 parts, vaselin 1 part.

236. Paraffin and Ether (*Marsh's method*[4]).—This method is similar to the chloroform process above described, the only difference being that *methylated* ether is employed as the solvent, instead of chloroform. The sections when cut are to be soaked in alcohol (why ?), and are then brought into a test-tube with a little more ether than is sufficient to cover the sections and heated to the boiling point of the ether. The ether is changed for fresh two or three times, and lastly alcohol is substituted for it. Marsh is of opinion that this method is "but a humbler substitute" for the freezing process. Why in the world ether should be taken instead of chloroform does not appear.

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[1] 'Zool. Anz.,' No. 129 (1883), p. 20.

[2] Ibid., No. 129 (1883), p. 21.

[3] Ibid., No. 130 (vol. vi), 1883, p. 51.

[4] 'Microscopical Section-cutting,' 2nd ed. (1882), p. 68.

237. Spermaceti and Castor-Oil (*Kleinenberg's method*[1]).—Four parts of spermaceti heated with 1 of castor-oil.

The objects are prepared by dehydration in alcohol, followed by soaking in oil of bergamot. The superfluous oil is removed from them, and they are well stirred about in the mass with a warm needle. The mass is removed from the sections by soaking for a few minutes in a mixture of 4 parts essence of turpentine with 1 of kreasote. The advantage of this mass is that it is more easily removed from the sections than other masses. It is an infiltration mass. Cut with a knife wetted with olive oil.

238. Spermaceti and Cacao-Butter (*Foster and Balfour's formula*[2]).—Four parts of spermaceti heated with one of cacao-butter.

Use as in Kleinenberg's method, *ante* No. 237.

239. Spermaceti, Castor-Oil, and Tallow (*Strasser's formula*[3]).—Spermaceti 4 parts, castor-oil 1 part, tallow 4 parts.

240. Transparent Soap Imbedding-mass (*Flemming's formula*[4]).—Take transparent soap (which must be free from glycerin, and it is therefore better to order from the maker a sample of *raw* soap, before it has been treated with glycerin and made up into toilet tablets), dissolve it by the aid of heat in $\frac{1}{3}$ to $\frac{1}{2}$ of its volume of *spiritus vini* (*not* absolute alcohol). Preserve for use after filtration in a corked vessel. Melt by warming when required. The object is prepared in alcohol: after imbedding the mass is set aside to dry thoroughly for one or two days. It is cut with a dry knife. The sections are washed out with water and mounted in glycerin. They may be stained after washing with carmine. (If they have been stained *before* imbedding with ammonia-carmine, the alkali of the soap will dissolve out the stain during the washing, but picro-carmine stains are more resistant.) The method succeeds best with osmium-preparations that have been hardened in alcohol. It is intended for the preparation of very delicate objects, such as chick-embryos.

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[1] 'Éléments d'Embryologie' (Foster and Balfour), pp. 296-8.

[2] Ibid., p. 296.

[3] 'Morph. Jahrb.,' v (1879), p. 243. 'Journ. Roy. Mic. Soc.' (N.S.), i, p. 840.

[4] 'Arch. Mik. Anat.,' ix (1873).

241. Transparent Soap (*Pölzam's formula* [1]).—The following account is taken from Salensky's paper on the gemmation of Salpa, l. c.

Take good white soap ("gewöhnliche Kernseife"), cut it up into thin slices, and put them to dry in the sun for some days, until they become white. The slices are then to be rubbed up to a fine powder, which is mixed with spirit to the consistency of porridge. Now mix the porridge with alcohol and glycerin in such proportions that the whole shall contain for every 10 parts by weight of the soap, 22 parts of glycerin, and 35 parts of alcohol (90 per cent,). Let the whole simmer until there is obtained a perfectly transparent, syrupy, somewhat yellow fluid.

The objects, previously dehydrated in alcohol, are imbedded in this mass in the usual manner.

The mass may be removed from the sections either by means of water or of very dilute alcohol.

Salensky considers that this is one of the best of imbedding masses, especially for embryological preparations. It was found to be the best of all for imbedding the stolon of Salps. It has the following advantages: 1. It is transparent. 2. It adapts itself perfectly to the objects. 3. It cuts remarkably well.

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[1] 'Morph. Jahrb.,' iii (1877), 3tes Heft, p. 558.

Objects may be stained either before imbedding or after cutting. Carmine and hæmatoxylin both stain well sections that have been cut in this mass.

242. Transparent Soap (*Kadyi's formula*[1]).—Twenty-five grammes of shavings of stearate of soda soap (any stearate of soda soap will do, but the most to be recommended is the sort known in commerce as "weisse Wachskernseife") are heated in a retort with 100 c.c. of 96 per cent. alcohol over a water-bath until the whole is dissolved. Filter if necessary. If a drop of the solution be now poured into a watch-glass it will be seen that it almost immediately solidifies into a *white* mass. This is not what is wanted, and is a sign that the solution does not contain water enough. Small quantities of water are therefore added by degrees to the solution, and the effect tested from time to time by pouring a drop of the mixture into a watch-glass. The mass will be seen to become more and more pellucid until a point is reached at which it is almost perfectly transparent, with merely the slightest blue opalescence. The preparation of the mass is then complete.

It is not possible to state *a priori* the exact proportion of water that should be added, as this naturally depends on the amount of water already present in the sample of soap taken. In very many cases it will be found that for about 120 g. soap solution, 5 to 10 g. of water will be required.

It is necessary to be very cautious in adding the water, as if too much be taken the mass solidifies more slowly or not at all. The greatest amount of elasticity and consistency is possessed by the mass at the moment in which it contains exactly the minimum amount of water necessary to make it transparent.

The reasons for this process are explained as follows. Stearate of soda soap is soluble in divers proportions in warm alcohol. On cooling, the solution either solidifies into a homogeneous and pellucid mass, or into a white granular mass; or, in certain cases, does not solidify at all. The result in each case depends on the proportion of water present in the solution. For instance, if 5 to 6 parts of a tolerably dry soap be dissolved in 100 parts of 96 per cent. alcohol, a solution is generally obtained that solidifies into a transparent mass. But such a mass is too soft, and its melting point too low; it melts by the heat of the finger. If now, in order to get a harder mass, you add more soap, you will get a solution that solidifies on cooling into a *white granular* mass; and it is only after adding to it a *certain* (*small*) quantity of water that you will obtain a solution that solidifies on cooling into a *transparent* mass. If you add more water than is just absolutely necessary to this end the mass will have too low a melting point, and will solidify more slowly; and if still more water be added the solution will not solidify for hours, or, indeed, not at all. The more soap you have in your alcoholic solution the more water *must* you add in order to get a transparent mass; and the more *may* you add without depriving the solution of the faculty of solidifying. Besides the mass prepared in the proportions given above, useful masses may be made for certain purposes with 10, 20, 30, 40 per cent., or more or less of soap in alcohol. Weisker has employed a mass composed of about equal parts by weight of soap and alcohol. Such a mass is transparent, but yellow and oily, and takes a long time to solidify. When cool it is very tough. It requires a considerable temperature to liquefy it, and has less penetrating power than the more alcoholic masses. It is, however, very suitable for hard, and especially for chitinous structures.

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[1] 'Zool. Anz.,' 37 (1879), vol. ii, p. 477.

The mass recommended above boils at about 60° to 70° C. Objects should be imbedded in it in a watch-glass or in paper cases in the usual way. Whilst cutting, wet the knife and the mass with strong alcohol (one advantage of this method is that the knife remains perfectly clean). The sections are brought into 96 per cent. alcohol, which frees them from the mass instantaneously if warmed, and after a time if left cold. Wash with fresh alcohol, stain, and mount. An *infiltration* method.

CHAPTER XXI.

CONGELATION IMBEDDING-MASSES.

243. Fresh tissues may be, and are, frequently frozen without being included in any mass, and in certain cases very satisfactory sections can be obtained in this manner. But the formation of ice-crystals frequently causes tearing of delicate elements, and it is better to infiltrate the tissues with a mass that does not crystallise in the freezing mixture, but becomes hard and tough. Gum-arabic affords such a mass. Some workers use common gum water, which is either poured into the well of the microtome or round the object on the object plate, according to the form of microtome used.

The following are Hailes's directions: "Perfectly fresh tissues may be cut without any previous preparation, using ordinary mucilage (acaciæ) to freeze in, but most specimens require special preparation. If preserved in Müller's fluid, alcohol, &c., they require to be washed several hours in running water; then, according to the suggestion of Dr D. J. Hamilton, the specimen is placed in strong syrup for twenty-four hours, and is removed to ordinary mucilage acaciæ for forty-eight hours and is then cut in the freezing microtome." The mucilage used is mucilage acacia (B. P.), which is poured into the well of the microtome, and the object placed in it.

244. Syrup and Gum Congelation Mass (*Hamilton's method*[1]).—Hamilton cuts sections (of hardened brain) in a Rutherford's freezing microtome. The hardening re-agent having been soaked out by water the tissues are prepared for freezing in the following manner, which it is important to observe, otherwise it will be found that the crystals of ice so break up the delicate nervous tissue as to render it totally useless for minute examination. The tissues are to be well soaked in syrup. The sugar somewhat retards the freezing, and besides, seems to alter the manner of crystallisation, so that instead of the ice being spicular in form it becomes granular and does no injury to the parts.

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[1] 'Journ. of Anat. and Phys.,' 12 (1878), p. 254.

The syrup requires to be of a particular strength, viz. double refined sugar, 2 ounces; water, 1 fluid ounce.

Wash the superfluous syrup from the surface, and put into the ordinary mucilage for an hour or so before cutting. Imbed in the freezing microtome with mucilage in the usual way. Float the sections into water.

245. Gum and Syrup Congelation Mass (*Cole's formula*[1]). —Gum mucilage (B. P.) 5 parts; syrup, 3 parts. (For brain and spinal cord, retinæ, and all tissues liable to come in pieces put 4 parts of syrup to 5 of gum). Add 5 grains of pure carbolic acid to each ounce of the medium.

(Gum mucilage (B. P.) is made by dissolving 4 ounces of picked gum acacia in 6 ounces of water.)

The syrup is made by dissolving 1 pound of loaf sugar in 1 pint of water and boiling.

This medium is employed for soaking tissues previous to freezing. They may remain in it for "any length of time; all the year round" if desired.

The freezing is conducted as follows: the gum and syrup is removed from the *outside* of the object by means of a cloth; the spray is set going and a little gum mucilage painted on the freezing-plate; the object is placed on this and surrounded with gum mucilage; it is thus saturated with gum and syrup, but surrounded when being frozen with mucilage only. This combination prevents the sections from curling up on the one hand, or splintering from being too hard frozen on the other. The mass ought to cut like cheese. Should freezing have been carried too far wait a few seconds.

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[1] 'Methods of Microscopical Research,' 1884, p. xxxix. 'Journ. Roy. Mic. Soc.' (N.S.), iv (1884), p. 318.

246. Gelatin Congelation Mass (*Sollas's method*[1]).—"Instead of gum one uses gelatin jelly. This is prepared and clarified in the usual manner. It should set into a stiff mass when cold. . . . The tissue to be cut is transferred from water to the melted jelly and should remain in it till well permeated."

The sections are transferred to a slide as soon as cut. On touching the glass they adhere to it. When enough sections have been thus arranged they are covered with a drop of glycerin; a cover is put on, and the mount closed with any suitable cement. In process of time the glycerin will permeate the gelatin and convert it into glycerin jelly; this may be hastened by placing the slide in an oven kept at about 20° to 30° C.

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[1] 'Quart. Journ. Mic. Soc.,' xxiv (1884), pp. 163-4. 'Journ. Roy. Mic. Soc.' (N.S.), iv (1884), p. 316.

CHAPTER XXII.

COAGULATION IMBEDDING-MASSES.

247. Egg Emulsion (*Calberla's formula*[1]).—The white of several eggs is separated from the yolk, the chalazæ are removed, and the white is cut up with a pair of scissors. Fifteen parts of the white are then mixed with 1 part of a 10 per cent. solution of sodic carbonate (10 parts of calcined soda to 100 of water) and briskly shaken. To the solution of albuminate of soda thus obtained the yolks of the eggs are now added and the whole is well shaken up. The mass is then poured into a deep vessel, allowed to settle for a short time, the foam and floating fragments of vitelline membrane are skimmed off with a strip of paper, and any larger pieces removed with forceps. The mass is then ready for use.

The objects to be imbedded are soaked in water for from three to ten minutes, in order to free them from the liquid in which they have been preserved. They are then soaked for from five to ten minutes in a portion of the egg mass placed in a shallow vessel (very delicate objects are soaked in common white of egg instead of egg mass). They are then fastened (with needles or otherwise) to the freshly-cut surface of a piece of egg mass that has previously been hardened by the process described below, and are then hardened by the same process. (If the objects are very small, the freshly-cut surface of hardened mass on which they are to be fixed is covered with a few drops of the emulsion, and the objects are arranged in this when it is nearly dry, which happens in five minutes' time. Very delicate objects are fastened as follows: thin slices of hardened mass are made, the alcohol that adheres to them is washed away with water, they are dried with blotting-paper and soaked for from ten to twenty minutes, or longer, in the freshly prepared mass. The objects to be imbedded are then arranged on a freshly-cut surface of old mass, as before directed, and are covered with one of the prepared slices of old mass; the slice is then fastened down with pins. It is sometimes advantageous to mount very delicate objects between two prepared slices of hardened mass, and then to fix the whole on the supporting block of hardened mass.)

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[1] 'Morph. Jahrb.,' Bd. ii, 3tes Heft (1876), p. 445.

The objects having been arranged in one of these ways on a block of hardened mass, the block is placed in a paper mould and fluid mass poured on to it to the height of at least 1½ to 2 centimetres above the objects. The whole is then brought into a capsule containing such a depth of 75 to 80 per cent. alcohol that the mass is immersed in it to one half its height or somewhat more. The capsule is then placed on a water-bath; is covered with an inverted funnel; and the whole heated to such a temperature that the alcohol does not quite boil. After half to three-quarters of an hour of this treatment the objects are brought into cold spirit, and the needles and all superfluous paper are removed. (On removal from the hot alcohol the mass ought to have "the consistence of gum.") They are then put away in alcohol of 85—90 per cent., which is changed for fresh at the end of twenty-four hours. After twenty-four hours more the mass is ready for cutting. (If the alcohol be changed three or four times during the first twenty-four hours, the mass will be hardened quicker. The longer the mass remains in the alcohol after forty-eight hours, that is, in the first change of alcohol, the better will be its consistency for cutting. But the alcohol should not be changed after forty-eight hours, except in the case of very resistant objects, such as chitinous structures, as further changing of the alcohol makes the mass too hard.)

Cutting is done with a knife moistened with alcohol.

The fluid mass may be preserved unchanged for a few days by placing in it a few lumps of camphor or thymol.

The advantages of this mass are that it enables very fine sections to be cut, that it gives to brittle objects the desired consistency, and that it enables the operator to place the object with perfect precision in any desired position. Calberla states that he has never observed any injurious effect of the high temperature (70° to 75° C.) on the objects.

This is not an infiltration-mass, according to Calberla's account. He recommends that if the preparations contain cavities, these should be filled with the fluid mass by injection before imbedding. But if the mass does not infiltrate the tissues, it is difficult to understand how it can render brittle objects "extraordinarily cuttable—*ausserordentlich schnittfähiger*." It is therefore probable that during the soaking in the fluid mass a certain superficial infiltration does take place.

248. Modifications of Calberla's Method.—This method of preparing the mass has been modified by other workers by the omission of the carbonate of soda treatment, the yolk and albumen being mixed by trituration for a few minutes in a mortar. The mass is then strained through linen to remove any solid fragments that may remain in it.

Objects are imbedded in the mass in paper trays. Small objects (ova) are imbedded in hollows scooped out on the surface of a small block of previously hardened mass. Hertwig fixes them in position in the following way. The hollow in the block is wetted with some freshly prepared fluid mass, the object is placed in position in it, a drop of absolute alcohol is then added, which coagulates the fluid mass, and the object remains fixed in position.

The objects having been imbedded in the trays are hardened either by the method of Calberla described above, or by heating for several days in alcohol steam of not more than

30° C. temperature,[1] followed by treatment for some days with cold alcohol.

Thoma states that it is very important that the temperature of the alcohol steam should not exceed 30° C., or innumerable air-bubbles will develop in the emulsion. A disadvantage of the process is that the mass cannot be easily detached from the sections, and we have no means of dissolving it in media which do not injure the objects. It is also generally necessary to stain the objects *in toto* before imbedding, as the mass stains in all the staining-fluids generally used, and becomes very visible in the preparations.

(*At Naples* both Selenka's and Calberla's methods *have been abandoned*, partly on account of the *aqueous nature* of the process, and partly on account of the long immersion in the warm liquid that is necessary.—AUTHOR.)

249. White of Egg (*Selenka's method*[2]).—The mass used is pure white of egg.

The object, previously freed from alcohol, which would cause the formation of bubbles, is soaked for an hour or for several hours, until it is thoroughly permeated, in white of egg. It is then imbedded in the mass in a paper tray. (*Strong* paper should be used for the tray, or bubbles may appear during the heating.) The tray is then heated in steam, or, still better, in dry air. For instance, the tray is placed in a small glass vessel (covered with a glass plate) set on a piece of wire netting over a water-bath. The water is kept boiling for some twenty minutes. The mass has by this time become hard enough to be ready for the next step in the process. It is thrown into strong spirit, which is changed once or twice in the course of one or two days, and for which absolute alcohol is at last substituted. After a few days the paper may be removed and the mass cut.

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[1] See the description and figure of Thoma's water-bath in 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 304. The water-bath is supplied by R. Jung, Mechaniker in Heidelberg, for 22 mark (=£1 2*s*.).

[2] 'Zool. Anz.,' 6 (vol. i, 1878), p. 130.

It is advantageous to clear the mass before cutting by soaking it for a day or two in clove oil or turpentine, notwithstanding that this process makes the mass somewhat softer, the perfect transparency imparted to the mass being advantageous for placing the object in position. The sections obtained may be mounted direct in balsam without freeing them from the mass, which under the microscope is almost or quite homogeneous.

If the mass should have become overhard in the alcohol, it may be softened by soaking in water (or to a certain extent in clove oil or turpentine).

"One advantage of this process is that it is not necessary that the objects should be previously hardened with so much care as is necessary for paraffin-imbedding." Selenka found the process applicable for siliceous and calcareous sponges, for worms, and for embryos of the fowl.

250. Albumen imbedding methods have the following common points. They are methods of imbedding by the *wet* way; that is, they do not require that the objects should be dehydrated and treated with chloroform, essential oils, ether, or other solvents of paraffin or collodion. The tissues retain their natural fatty and aqueous constituents, and this, from a histological point of view, is in many cases an important advantage. Another good quality of these methods is that they allow of the cutting of very thin and perfect sections from the most friable objects. Their great defect is that no means have been found for removing the mass from the sections when cut. Its presence in the tissues renders subsequent staining extremely difficult or impossible (as the albumen takes on a stain with most of the usual colouring agents, and does not always yield it up on washing out), and, which is worse, produces confusion in the microscopic images and introduces causes of error into the interpretation of them.

CHAPTER XXIII.

EVAPORATION IMBEDDING-MASSES (GUM, GELATIN, COLLODION, ETC.).

251. Gum and Glycerin (*Joliet's formula*[1]).—Pure gum arabic dissolved in water to the consistency of a thick syrup. (Solutions of gum sold under the name of strong white liquid glue (? colle forte blanche liquide à froid) may also be used; they have the advantage of having a uniform consistency.) Pour a little of the solution into a watch-glass, so as not quite to fill it, add from 6 to 10 drops of pure glycerin, stir until thoroughly mixed.

Between the limits of 6 to 10 drops of glycerin the proportions most suitable to the nature of the object and to the season of the year must be found by experimental trials. In the winter or in rainy weather less glycerin should be taken than in the summer or dry weather.

It is often well to soak the object in glycerin before putting it into the mass. In this case less glycerin should be added to the gum, in proportion to the amount of glycerin contained in the object.

The object is imbedded in the mass in the watch-glass, and the whole left to dry for from one to four days. When it has assumed a cartilaginous consistency, a block containing the object is cut out, turned over, and allowed to dry again until wanted for use. A stove, or the sun, may be employed for drying, but it is best to dry slowly at the normal temperature. The block may be preserved in good condition almost indefinitely, the gum, when mixed with a sufficient quantity of glycerin, never becoming hard or brittle. It is generally better to wait till the blocks have assumed such a consistency that they cannot be easily bent. It is after having waited almost a week that the author always obtained the best sections. The gum is dissolved out from the sections by means of a drop of water on the slide. The sections are then covered, and a drop of glycerin being added, the preparation is complete as soon as the water has evaporated.

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[1] 'Arch. Zool. exp. et gén.,' x (1882), p. xliii. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 890.

An infiltration-mass. It has the advantage of being transparent. Joliet employs it for *Pyrosoma*. A similar mass was employed by Hertwig for *Ctenophora* ('Jen. Zeitsch.,' xiv (1880), pp. 313—314; 'Journ. Roy. Mic. Soc.' (N.S.), ii, p. 278).

It would probably be advantageous to add some preservative substance to this mass.

252. Gum-Imbedding (*Stricker's method*[1]).—A concentrated solution of gum arabic. The object may be prepared in alcohol and imbedded in the gum in a paper case. The whole is thrown into alcohol, and after two or three days may be cut.

(It is better to add a little glycerin to this mass.—AUTHOR.)

253. Gelatin (*Kaiser's formula*[2]).—One part by weight of the finest French gelatin is left for about two hours in 6 parts by weight of water; 7 grammes of glycerin are added, and for every 100 grammes of the mixture 1 gramme of concentrated carbolic acid. The whole is warmed for ten to fifteen minutes, stirring all the while, until the whole of the flakes produced by the carbolic acid have disappeared. Filter whilst warm through the finest spun glass which has been previously washed in water and laid whilst wet in the filter.

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[1] 'Hdb. d. Gewebel.,' p. xxiv.

[2] 'Bot. Centralb.,' i (1880), p. 25. 'Journ. Roy. Mic. Soc.,' iii (1880), p. 504.

The objects should have a warm bath in the mass until penetrated by it. They may be cut in the mass as soon as cool, if they are of a suitable consistency, or the mass may be treated with absolute alcohol for from ten to thirty minutes, by which means any degree of hardness may be imparted to it. The mass is removed from the sections by washing on the slide with a fine jet of warm water.

A transparent infiltration-mass.

254. Glycerin-Isinglass (Glycerin Jelly) (*Klebs' formula*[1]). —A concentrated solution of isinglass is mixed with half its volume of glycerin. This mass may be hardened if desired in either alcohol or chromic acid.

255. Collodion Imbedding-mass (*M. Duval's method*[2])—Collodion appears to have been first employed (in the dry state) by Latteux ('Manuel de technique Microscopique,' p. 236), in order to obtain sections of hairs. If, however, it be desired to employ it for imbedding delicate structures, such as embryos or blastoderms, it will be found that the collodion in drying contracts so strongly as to cause distortion of the imbedded structures. This may be avoided by the following process:

The objects are hardened in the usual way; they are stained *in toto* (if this be considered more convenient than staining the sections when made), and are soaked in alcohol. They are then plunged for a few minutes in ether and brought into normal collodion (free from castor-oil), where they remain till thoroughly permeated. (From ten minutes to twenty-four hours if there be cavities that it is desired to fill and support.) They are taken out of the collodion and thrown into alcohol of 36°. (If the objects are small and delicate, so as to require support during cutting, they are placed on a piece of pith to which they will adhere on being put into the alcohol.) The alcohol immediately causes the collodion to solidify, *without contraction*, into an elastic mass which supports every part of the contained structures. Sections may then either be cut at once, or the objects may be left in the alcohol till wanted for any length of time without further change.

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[1] 'Arch. Mik. Anat.,' v (1869), p. 165.

[2] Robin's 'Journal de l'Anat. et de la Phys.,' 1879, p. 185.

The sections are cut with a knife wetted with 36° alcohol and are floated into water or on to the slide.

If the object was not previously stained *en masse*, the sections may now be stained by carmine or picro-carmine in the usual way. *There is no need to remove the imbedding-mass*, as it does not take the stain.

They may be mounted simply in glycerin; the collodion will remain as clear as glass. Duval found that he could not mount them in balsam or dammar as these substances made the collodion "opaque and granular." (I am unable to understand this, as I have long mounted mine in balsam or dammar, and found them remain perfectly transparent. Duval does not state what clearing agent he employed for dis-alcoholisation.) He obtained, however, satisfactory results by dehydrating *rapidly* with absolute alcohol (*absolute* alcohol dissolves collodion in a short time, and a delicate section would thus be deprived of the support necessary to hold its parts together), adding (on the slide) a drop of oil of cloves, (which *entirely* dissolves the collodion) and closing the mount with chloroform-balsam.

256. Collodion-Imbedding (*the Author's method*).—Having for many months employed the collodion method with most satisfactory results, I shall probably do well to relate here the details of any practice. I dehydrate my objects with alcohol, soak them in ether, put them into a porcelain mould, and cover them with collodion. I then cover the mould with a shade, and leave it for a few hours. As soon as the collodion (of which only just enough to cover the object should have been taken) has so far shrunk that the object begins to lie dry, a drop of collodion is added, and the whole left as before. This process is repeated every few hours for two or three days, at the end of which time it will generally be found that the object is imbedded in a magma of half-dry collodion of sufficient hardness for section-cutting. The mass is then scooped out of the mould and preserved till wanted in 70 per cent. or 80 per cent. alcohol. (Soft tissues *do not shrink* under this treatment. I have obtained by this means admirable sections of the most delicate sponges, for instance.) I cut with a knife wetted with 70 per cent. alcohol, and float my sections into a watch-glass with 70 per cent. alcohol, which is gradually changed for stronger up to 90 per cent. (Stronger must not be used.) I then clear as rapidly as possible with oil of cloves or carbolic acid, and mount in balsam. If the dehydration with 90 per cent. alcohol and the clearing with clove oil be performed rapidly, the mass will not dissolve out; it softens and becomes perfectly invisible, and I believe it is this which may have led Duval to believe that it was dissolved, whilst in reality its presence was only masked by the identity of its refractive index with that of the clearing medium.

Objects should be stained *en masse* before imbedding unless the sections are to be fixed and stained on the slide.

I consider that there is only one defect in this method, and that is the impossibility of obtaining a mass hard enough to admit of the cutting of the very thinnest sections, without causing shrinkage in the specimen. For the point at which the drying-up must be arrested in order that the tissues may not suffer, is one at which the magma of collodion is not yet sufficiently hard from the mechanical point of view of section-cutting. This defect in part is eliminated by the use of a special collodion, viz. Schering's "celloidin" (*see* below). I should mention that I was led to the method of partial and gradual drying-up, by finding that when I followed Duval's instructions to throw the imbedded objects at once into 36° alcohol, I was unable to obtain a sufficiently resistant mass. It is possible that the collodion I employed was not of a suitable nature.

257. Celloidin.—Celloidin is a preparation of pure pyroxylin, patented for Germany and England under the name of "Schering's celloidin." It is manufactured by the *Chemische Fabrik auf Actien* (vorm. E. Schering), Berlin, N. Fenstrasse, 11, 12. It may be obtained through the post by writing to Schering's Grüne Apotheke, Wittick and Benkendorf, Berlin, N. Chaussée-Strasse, No. 19.

It is stated to be prepared with the purest pyroxylin, and to be always of a uniform composition. It is sent in the form of tablets of a tough, gelatinous consistency and slightly milky-white transparency. These tablets have exactly the consistency that is required for section-cutting. They contain 20 per cent. of pure pyroxylin. Celloidin is entirely soluble, in all proportions, in ether and alcohol. It is free from acids. It is not detonant. If ignited it burns like paper; heated in a test-tube it carbonises without exploding.

In order to make a 2 per cent. collodion, take one tablet of celloidin (which contains 40 grammes of the dry pyroxylin) and such a quantity of alcohol and ether that the whole shall weigh 2000 grammes. For a 3 per cent. collodion you take such a quantity of alcohol and ether that the whole shall weigh 1333 grammes; and for a 4 per cent. collodion such a quantity that the whole shall weigh 1000 grammes. The relative portions of alcohol and ether may be taken according to discretion. To prepare a medicinal collodion according to the Prussian pharmacopœia, you take for each tablet 720 grains of ether and no alcohol, as the celloidin already contains the prescribed proportion of alcohol.

The tablets cost three marks (= three shillings) each. A single tablet would, I think, suffice for imbedding many hundreds of embryos.

258. Celloidin Imbedding-mass (*Merkel and Schiefferdecker's method*[1]).—Struck with the advantages of a collodion imbedding-mass as recommended by Duval (Method No. 255), the authors have followed up the clue, and find in celloidin a substance that can be obtained of any degree of concentration that is desired, and that "performs more, is less hurtful to tissues, and is easier to manage than any other known imbedding-mass."

Celloidin is cut into small pieces and dissolved in equal parts of absolute alcohol and ether. The tissues to be imbedded are thoroughly soaked in absolute alcohol, from which they are brought into the celloidin (in a well-closed vessel), and remain there until thoroughly soaked (from a few minutes to eight days or more, according to thickness). If the objects contain cavities that it is desired to fill, it is best to use a thinner, and therefore more penetrating, solution of celloidin. When soaked remove the preparations to a paper-tray (or simply a small piece of leather), surround them with celloidin, wait a few minutes until a skin has formed on the celloidin, and throw them into alcohol of 82° Richter (a considerable quantity of alcohol should be taken). After twenty-four hours the preparations on leather are generally fit to be cut, whilst those in paper-trays may have the paper removed and be put back into the alcohol for twenty-four hours more.

The preparations may remain in the alcohol for any length of time without harm.

Sections are cut with a knife moistened with common alcohol; they are floated either into water or alcohol.

Staining.—Without any further manipulation they may now be stained with the usual staining agents, just as if they were not imbedded, so, *e.g.* with carmine or hæmatoxylin. Some of the anilin colours, however, behave somewhat differently, eosin, for example, so that an eosin-dahlia double stain is not obtainable.

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[1] 'Arch. Anat. u. Phys.' (Anat. Abth.), 1882, p. 200.

Clearing and mounting.—Sections may be cleared in glycerin, which suffices to make the celloidin as transparent as glass.

For a balsam mount proceed as follows: Dehydrate with 95 per cent. alcohol (not absolute, or the celloidin will be dissolved), clear with oil of bergamot, sandalwood, or origanum (the last by preference), and mount in balsam or castor-oil. Oil of cloves should *not* be employed, because it dissolves the celloidin.

Schiefferdecker states that the celloidin imbedding-mass gives to tissues a firmness sufficient to enable sections of 0·01 mm. to be obtained with a Jung's "Schlittenmikrotom."

(Notwithstanding the advice of the eminent authorities above quoted, I have persisted in clearing my sections with clove oil, and find that I obtain perfect preparations.)

259. Celloidin-Imbedding (*Thoma's process*[1]).—It has been found by some workers (and I can confirm the fact) that the method of imbedding described above by Schiefferdecker frequently gives rise to the formation of air-bubbles in the mass. In order to obviate this defect Thoma recommends a process of gradual evaporation, such as that which I have recommended above for common collodion-imbedding. You imbed in a paper-box and cover with a shade, and after a few days, when the mass has shrunk a ltttle, moisten it with a drop of ether and add more celloidin solution (if necessary). After a few days the mass will have gained sufficient consistency to allow of the removal of the paper, and may be thrown into "very dilute" alcohol, which will complete the hardening in a few days.

Another process described by Thoma is as follows (l. c.): "The even surface of a cork is covered with a thick solution of celloidin so as to form by evaporation a strong collodion membrane on the cork. Upon this is put the specimen, covered layer by layer with fresh quantities of the solution of celloidin, each being allowed to dry only partially. When the object is thoroughly covered immerse it in alcohol of 0·842 sp. gr. In twenty-four hours the whole is ready for cutting.

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[1] 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 305.

I have tried all these methods, and am at a loss to say which give the best results. For small objects I have also employed the following plan with fair results. Imbed on a cylinder of pith, and set to harden in alcohol vapour by placing the cylinder of pith in a stoppered bottle containing a small quantity of alcohol, not enough to wet the specimen.

The difficulty in all these cases is to prevent the superficial layer of celloidin from hardening much more quickly than the internal layers.

It would be highly advantageous to discover some means of performing the process of imbedding under the surface of alcohol.

260. Collodion or Celloidin-Imbedding (*Viallanes' method*[1]).—The dehydrated objects are soaked in ether, for some hours, and then brought into the collodion, in which they ought to remain for several days. They are then brought into *chloroform.* "Under the influence of this reagent, the collodion coagulates into a mass having the consistence of wax, but having also an elasticity that renders it unbreakable, and having besides the precious quality of being admirably transparent, and possessing exactly the index of refraction of glass."

Dr J. Barrois informs me that this method gives results greatly superior to those obtainable by hardening colloidin in alcohol. He proceeds as follows. The object, previously soaked in ether, is placed in a test-tube of convenient size and covered with collodion. As soon as a sufficiently strong film has formed on the collodion, *i.e.* in a few minutes, the tube is filled up with chloroform, and left for two or three days. By this time the collodion mass will be considerably hardened, and also somewhat shrunk, so that it can be shaken out of the tube. It is then brought into fresh chloroform in a larger vessel, where it remains for about six days, after which time it is generally ready for cutting.

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[1] 'Recherches sur l'Hist. des Insectes, &c.,' Paris, 1883, p. 129.

Good chloroform is a necessity, as the reaction cannot be obtained with samples of chloroform that are not free from water.

Since the above directions were written, I have experimented with chloroform, and find that, as stated, it is vastly superior to alcohol for hardening the collodion. In some cases, a few *hours'* immersion is sufficient to give the requisite consistence. In no case did my specimens require more than three days. But the length of time required varies in a very inexplicable way, so that no rule can be given. The collodion frequentlybecomes opaque on being put into the chloroform, but regains its transparency after a time.

261. Shellac-Imbedding (*Hyatt's method*[1]).—Prepare the object by soaking in alcohol, and then put it for a day or two into a clear alcoholic solution of shellac. Take a cylinder of soft wood, split it, and make a groove in one or both of the half cylinders sufficiently large to admit the object without pressure. Imbed in the groove with plenty of thick shellac solution and tie together the two halves of the cylinder with thread. In a day or two the shellac will be quite hard; the cylinder is then fixed in a microtome, is soaked with warm water, and sections made. Should the shellac prove so opaque as to interfere with a proper examination of the sections, a drop of borax solution will immediately remove this difficulty.

This process is intended for the purpose of making sections through hard chitinous organs consisting of several pieces, such as stings and ovipositors, retaining all the parts in their natural positions.

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[1] 'Am. M. Micr. Journ.,' i (1880), p. 8. 'Journ. Roy. Mic. Soc.,' iii (1880), p. 320.

262. Copal-Imbedding (*von Koch's method*[1]).—Small pieces of the object are stained in the mass and dehydrated with alcohol. A thin solution of copal in chloroform is prepared by triturating small fragments of copal in a mortar with fine sand, pouring on chloroform to the powder thus obtained, and filtering. The objects are brought into a capsule filled with the copal-solution. The solution is now slowly evaporated by gently heating the capsule on a tile by means of a common night-light placed beneath it. As soon as the solution is so far concentrated as to draw out into threads that are brittle after cooling, the objects are removed from the capsule and placed to dry for a few days on the tile, in order that they may more quickly become hard. When they have attained such a degree of hardness that they cannot be indented by a finger-nail, sections are cut from them by means of a fine saw. The sections are rubbed down even and smooth on one side with a hone, and cemented, with this side downwards, to a slide, by means either of Canada balsam or copal-solution. The slide is put aside for a few days more on the warmed tile. As soon as the cement is perfectly hard, the sections are rubbed down on a grindstone, and then on a hone, to the requisite thinness and polish, washed with water, and mounted in balsam.

The process may be varied by imbedding the objects unstained, removing the copal from the sections by soaking in chloroform, decalcifying them if necessary, and then staining.

It is sometimes a good plan, after removing the copal, to cement a section to a slide by means of hard Canada balsam, then decalcify cautiously the exposed half of the specimen, wash, and stain it. In this way, von Koch was able to demonstrate the most delicate lamellæ of connective tissue in *Isis elongata*.

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[1] 'Zool. Anz.,' 2 (vol. i, 1878), p. 36.

This method was imagined in order to enable the hard and soft parts of corals to be studied in their natural relations. It is evidently applicable to the study of any structures in which hard and soft parts are intimately combined. It is very highly spoken of by those who have worked with it.

263. Semi-pulped Paper Imbedding-method (*Richardson's method*[1]).—The object is prepared by soaking in water. Strips eight or nine inches long are cut from white unglazed printing paper, dipped in water, drained rapidly, and rolled round the object as tightly as may be without tearing the paper. Enough paper should be rolled round the object to form a plug requiring a little pressure for sending it home in the well of the microtome. The diameter of the object should be, if possible, one quarter of an inch less than that of the microtome-well. Wet the mass with water whilst cutting, and float the sections into water. The tissues to be cut must neither be very hard nor very soft.

264. Porous Gelatin or Artificial Pith for Section-Cutting (*Mayer's formula*[2]).—Take tablets of pure gelatin, soak them in water until they swell, then dissolve by means of heat, add a quarter to one half in volume of castor-oil, and shake well together. When on the point of cooling, pour the emulsion into a capsule. Extract the castor-oil by means of 90 per cent. alcohol, and the gelatin remains as a finely porous mass, an artificial pith. It must not be kept exposed to the air, or it will become soft.

The object of thus preparing the gelatin is to get rid of its too great elasticity, and thus give it a consistency that makes it a most fitting support on which objects to be cut may be fixed. They are fixed on it by means of warm gelatin-solution.

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[1] 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 474.

[2] 'Mitth. Zool. Stat. Neapel,' ii (1881), p. 27.

CHAPTER XXIV.

SERIAL SECTION MOUNTING.

265. One of the most important of the improvements that have of late years been effected in histological practice, consists in the invention of methods by means of which sections (or other specimens) are fixed in position on the slide before being mounted, thus enabling the operator to mount a *series* of sections or other objects under the same cover; and, what is perhaps even more important, rendering it certain that no loose parts of the preparations be moved ever so little from their natural positions during the processes of removing the imbedding-mass, clearing, and mounting. Before the invention of these methods mounting processes were precarious; they have now become well-nigh infallible. It is now possible to cut and mount in serial order many hundreds of sections without losing or displacing one, or any part of one.

The principle of these methods is to fix the sections in position on the slide by means of a transparent cement that is not soluble either in the menstruum used for dissolving-out the imbedding-mass or in the clearing medium. Gum-arabic had been used for attaching objects to the slide before mounting (a practice emanating from the diatomists); but its use did not readily fit in with the process of removal of imbedding-mass from sections and other treatment of them before mounting. The first step in advance was taken by Giesbrecht in 1881; he arranged his sections on a sticky film of shellac, which is insoluble in the turpentine that he employed for removing the paraffin in which he imbedded, and for clearing the sections. The method was a most important step in advance, but it was not infallible. It had the great disadvantage of not allowing of the manipulation of the sections on the slide, so that if they fell into the wrong position, or fell folded, they must so remain; wrinkles could not be smoothed out. This defect was obviated by the process recommended about the same time by Gaule. A slide was moistened with alcohol, and the sections arranged and flattened out with a brush moistened with alcohol; they were then melted on to the slide by heating, and the paraffin in which they were imbedded was extracted by xylol, either carefully dropped on them from a pipette, or allowed to run under the cover, which was put on dry in order to keep the sections in position by its pressure. But this, too, was not infallible, whilst at the same time it was frequently felt to be a defect in both these methods that they did not allow of staining the sections after cutting. This defect is removed by the elegant process published in 1883 by Frenzel. A film of gutta percha is made by allowing a thin layer of gutta-percha solution to dry on a slide. The sections are arranged smoothly on it by brushing them out with alcohol (thus removing the first of the defects of Giesbrecht's method). The slide is then slightly heated, the gutta percha becomes sticky and fixes the sections firmly in position. When cool the imbedding-mass is extracted by a suitable solvent, and the sections may then be passed through successive alcohols and stained without any risk of their being disturbed in the process. Frenzel at first used hot absolute alcohol for extracting the paraffin used for imbedding, but this was not quite satisfactory, and the method was brought to its present state by the discovery of Threlfall, in the same year, that naphtha is a suitable solvent for the paraffin, which it readily dissolves without attacking the gutta percha rapidly enough to loosen the sections, whilst at the same time it can either be removed by alcohol if it be desired to stain, or the sections may be mounted from it direct in balsam.

A simple modification (*see* below, No. 270) adapts the process to the case of celloidin objects.

The methods of Schällibaum and Mayer possess almost all the advantages of the Frenzel-Threlfall method, and are somewhat more convenient in practice. The Frenzel-Threlfall method has the peculiarity of offering a *dry* film, on which wrinkles may be smoothed out from large sections by brushing with alcohol, which cannot be well done on a sticky film; this is an advantage for such objects. But for small sections that are not likely to require smoothing a sticky film is preferable, as it removes the risk of light sections being blown off the slide by a sudden draught, or by the breath of the operator, as sometimes happens.

266. Shellac Process for Section-fixing (*Giesbrecht's method* [1]).—Prepare a stock of slides covered with a thin and even film of shellac. This is done as follows: Make a not too strong solution of brown shellac in absolute alcohol, filter it thoroughly; warm the slides, and spread over them a layer of shellac by means of a glass rod dipped in the solution and drawn once over each slide. Let the slides dry.

Just before beginning to cut your sections take a prepared slide and brush it over *very thinly* with kreasote applied by means of a brush; this forms a sticky surface on which the sections are now arranged one by one as cut, care being taken to bring them on to the slide with as little surrounding paraffin as possible.

When all the sections are arranged the slide is heated on a water-bath for about a quarter of an hour at the melting point of the paraffin; this causes the paraffin to run down into a thin layer, and allows the sections to fall through it and come into close contact with the shellac film, whilst at the same time it evaporates the kreasote.

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[1] 'Zool. Anz.,' No. 92 (vol. iv, 1881), p. 484.

The slide is allowed to cool, and the sections are now found to be firmly fixed in the shellac. The paraffin is dissolved away by dropping turpentine on to the sections, which are then mounted in Canada balsam. There is no danger of the sections being floated away by the turpentine, because turpentine does not dissolve shellac.

In the note in the 'Zool. Anz.' above quoted, the shellac solution is stated to be prepared with common brown shellac (choosing, of course, by preference the paler sorts), on account of the insolubility of white shellac in alcohol. In the 'Mitth. d. Zool. Stat.' of Naples, of the same year, "bleached white shellac" is recommended to be dissolved as before in absolute alcohol. In the 'Journ. Roy. Mic. Soc.' (N.S.), vol. ii. (1882), p. 888, it is stated (on whose authority is not clear) that the solution is made by mixing 1 part of bleached shellac with 10 parts absolute alcohol, and filtering. In the same place it is added that "Dr Mark uses the bleached shellac in the form in which it is prepared for artists as a 'fixative' for charcoal pictures. It is perfectly transparent, and a film of it cannot be detected unless the surface is scratched. He attaches a small label to the corner of the slide, which serves for the number of the slide and the order of the sections, and at the same time marks the shellac side otherwise not distinguishable." (The latter object is better attained by gumming a paper square, or spinning a ring with ink, in the centre of the unprepared surface of the slide. The disk or ring then serve at the same time for centering the group of sections.—AUTHOR.)

The account given in the 'Mitth. d. Zool. Stat.,' further varies in one other detail from that given in the 'Zool Anz.' It directs that the shellac slides be brushed before cutting with *oil of cloves,* instead of kreasote, the slide being slightly warmed before brushing.

267. Shellac-fixing solution (*Caldwell's formula*[1]).—"A strong solution of shellac in anhydrous kreasote." (Warm the kreasote to make the solution.) "Care must be taken to have as little as possible on the slide."

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[1] 'Quart. Journ. Mic. Soc.' (N.S.), lxxxvii (1882), p. 336.

Bourne notes that the slides should be heated for at least half an hour at a temperature two or three degrees above the melting-point of the paraffin; and that the turpentine should be flooded on to the sections from a small pipette *while the paraffin is still molten.* This dissolves melted paraffin instantaneously, and precipitates the shellac fastening the sections to the slide. The turpentine is allowed to flow off and is replaced by new, until all the paraffin is removed. The slide is then drained, wiped, and the cover put on. Some very fluid balsam is previously put on the cover-glass, which is turned over and quickly lowered. *The balsam dissolves the shellac,* so that if the cover be not quickly lowered the sections may shift or delicate sections come to pieces and float over the slide.

268. Paraffin and Xylol Section-fixing Methods (*Gaule's section methods* [1]).—Sections are cut from a paraffin-imbedded object in the usual manner. The point now is, to dissolve away the paraffin from the sections without floating away any part of the structures from its natural position.

A slide is moistened with alcohol, the sections are arranged in it by means of a camel-hair brush, also moistened with alcohol; the slide is slightly warmed so as to cause the section to stick to the slide; a cover is put on, and a solution of Canada balsam in xylol (equal parts of each) run underneath it. If the sections are not thicker than $\frac{1}{70}$th mm., they will be cleared at once, and nothing remains but to refill the cell day by day as the xylol evaporates, in order to have a perfect mount. If, however, the sections are thicker than $\frac{1}{70}$th mm., they will contain more paraffin than the xylol balsam can dissolve. In that case, the excess of paraffin must be removed by means of a drop of pure xylol (the sections being first melted on to the slide as before), and the mount is completed by means of xylol balsam.

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[1] 'Arch. Anat. u. Phys.' (Phys. Abth.), 1881, p. 156.

Both the moistening with alcohol, and the heating, are necessary for the attachment of the sections to the slide; the effect is not obtainable by means of one of these manœuvres alone.

269. Gutta-percha Section-fixing Process (*Frenzel's first method*[1]).—Dissolve gutta percha in chloroform + benzin, allow the solution to settle, and filter until clear and almost colourless. (The filtrate should be allowed to stand two or three weeks, be frequently well shaken, and any precipitate that may form at the end of that time be removed by filtration. An excellent 1 per cent. gutta-percha solution may be obtained from Ferd. Beyrick, Berlin, N. Linienstrasse, 114.) The solution *should not be too thin;* it should flow but slowly on the slide.

The slides are prepared by *brushing* with the solution, and allowing to dry.

Paraffin sections are arranged on the prepared surface, and smoothed out, if necessary, by treating them with absolute alcohol. They are then exposed for five or ten minutes (or even a few seconds) to a temperature of 35° to 50° C. (in no case higher than 55°). This makes the gutta-percha film sticky and fastens the sections. Allow the slide to cool. When cool, pour over the sections *ample quantities* of naphtha, allowing the naphtha to flow away *quickly, until the sections appear almost dry.* You can now remove the naphtha with absolute alcohol, and pass through successive alcohols for the purpose of staining; or you can mount direct in balsam. Or, in the case of very small sections, you can take a further precaution. Allow the naphtha almost to evaporate, pour a few drops of gutta-percha solution over the sections, and allow the gutta percha to dry before bringing them into alcohol. Staining succeeds perfectly in this case, as the gutta percha has not time to penetrate the tissues, but merely forms a film over the sections that does not prevent them from being penetrated by the staining fluid.

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[1] 'Zool. Anz.,' No. 130 (vol. vi, 1883), p. 51, and No. 145, p. 423.

The employment of naphtha in the above process was suggested by Threlfall. In the first form of his process Frenzel extracted the paraffin by putting the slides into a vessel with absolute alcohol warm (40° to 50° C.). If sufficient quantities of alcohol be employed, the paraffin will be extracted in five to fifteen minutes; but large quantities must be employed, as alcohol only dissolves a small proportion of paraffin. When the alcohol has become saturated, it may be filtered *cold*, and used over again.

But since he has experimented with naphtha as recommended by Threlfall, he has abandoned the alcohol process.

Frenzel considers gutta percha a better medium than the caoutchouc recommended by Threlfall. The advantages of caoutchouc are, that it more readily makes a good solution, and that the solution dries quicker on the slides. But gutta percha is preferable, because it forms a stickier film, since it never becomes quite dry, and softens easily with warmth; and also because it is less quickly attacked by the solvents of paraffin, such as naphtha, than caoutchouc is.

270. Gutta-percha Section-fixing Process (*Frenzel's second method*[1]).—A modification of the above method adapted to the case of objects imbedded in celloidin. The sections are arranged on the gutta-percha film prepared as above directed, and wetted with benzine or chloroform. This causes them to adhere to the gutta percha. They are allowed to dry, and treated as may be desired, either by staining or otherwise. The celloidin may be dissolved out before mounting by means of clove oil if desired.

This process allows of perfectly satisfactory staining.

271. Caoutchouc Section-fixing Process (*Threlfall's me-*

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[1] 'Zool. Anz.,' No. 130 (vol. vi, 1883), p. 52.

thod[1]).—Slides are prepared with films made by pouring over them a thin solution of raw caoutchouc (india rubber) in benzin or chloroform. Proceed as in Frenzel's gutta-percha process, removing the paraffin with naphtha, or any light paraffin oil, the solvent action being more rapid the lower the boiling point used. All the rest of the treatment as in Frenzel's method.

Threlfall was led to adopt caoutchouc instead of gutta percha by finding that the gutta percha was appreciably soluble in the paraffin oil used for removing the imbedding-mass. The recommendation is not adopted by Frenzel, for the reasons given above (No. 269).

272. hellac and Gutta-Percha Section-fixing Process (*Frenzel's method*[2]).—The sections are fixed by Giesbrecht's shellac method or by means of gum-arabic. The paraffin is extracted by means of turpentine, the turpentine is allowed to evaporate, or is washed out with chloroform. The sections are then covered with a few drops of gutta-percha solution, which is allowed to dry somewhat before bringing them into alcohol for the purpose of staining.

A lengthy proceeding, but *absolutely sure.*

273. Arabin for Fixing Mounts (*Waddington's formula*[3]).—For attaching objects to the slide, Waddington uses arabin, a purified gum-arabic, which has the advantage of not presenting a granular appearance under the microscope as ordinary gum-arabic does.

Dissolve clear and white gum-arabic in distilled water to the consistency of thin mucilage. Filter. Pour the filtrate into rectified alcohol, and shake well; the arabin separates as a white pasty mass. Place it on filter paper and wash with pure alcohol until the washings are free from water. Dry.

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[1] 'Zool. Anz.,' No. 140 (vol. vi, 1883), p. 301.

[2] Ibid., No. 150, p. 424.

[3] 'Journ. Quek. M. Club,' vi (1881), p. 199. 'Journ. Roy. Mic. Soc.' (N.S.), i (1881), p. 704.

The white powder thus obtained should be dissolved in distilled water and filtered twice. It may then be placed on slides, which are drained, dried, and put away till wanted. In this condition it may be preserved indefinitely.

274. Gum-Arabic Section-fixing Process (*Flögel's method*[1]). —Make a solution of one part gum-arabic in twenty parts water, filter, and add a little alcohol to prevent the formation of mould. Slides are prepared by pouring the solution over them, and draining. (It is important that the slides be so perfectly clean as to be evenly wetted all over by the gum solution.) Sections may now be cut and laid on the gum surface before it has become dry, and floated into the proper position; this is the best plan for sections of $\frac{1}{100}$ mm. thickness, and for large sections. For thinner and small sections it is best to take slides that have completely dried, arrange the sections dry on the gum film, and then breathe on it until the gum has become sticky.

If only a few small sections are to be mounted under one cover, it is not necessary to remove the paraffin before mounting; the balsam will suffice to dissolve it. If 50 or 100 sections are to be mounted under one cover, the paraffin should be extracted by benzin and balsam applied before the benzin has evaporated.

Flögel says that in these processes "practice makes perfect."

275. Collodion Section-fixing Process (*Schällibaum's method*[2]).—" One part of collodion is mixed with three to four volumes (according to its consistence) of oil of cloves or lavender oil and well shaken. The clear solution is spread with a brush over the slide in a thin layer. . . . The sections are arranged on it before the collodion has become dry, and the oil of cloves is evaporated by gentle heat over a water-bath, which takes five to ten minutes. The sections thus fixed can be treated for days with oil of turpentine, chloroform, alcohol, and water, without losing their adhesion. Stain as desired. If any cloudiness should appear between the sections, through the solution having been too concentrated or laid on too thick, it may be removed by passing a brush wetted with oil of cloves several times between the sections."

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[1] 'Zool. Anz.,' No. 151 (vol. vi, 1883), p. 565.

[2] 'Arch. Mik. Anat.,' xxii (1883), p. 689. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 736.

276. White of Egg Fixing Medium (*Mayer's formula*[1]).—A mixture of equal volumes of filtered white of egg and glycerin, to which are added a few drops of some antiseptic (carbolic acid). A thin layer of the mixture is spread on a cold slide with a fine brush and the sections laid on it, and warmed for some minutes on a water-bath. As the paraffin in the sections melts it carries the albumen away from them, and this is one of the advantages of the method. The sections may be treated with turpentine, alcohol, and aqueous or other stains without any danger of their moving.

The function of the glycerin is merely to keep the layer of albumen moist.

Mayer considers that this method is an improvement on those of Frenzel, Threlfall, and Schällibaum. It allows of the staining of sections on the slide with anilin stains, which is seldom practicable with Schällibaum's method, as the collodion stains with most anilin stains, and does not yield up the colour to alcohol.

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[1] 'M. T. Zool. Stat. Neapel,' iv (1883). 'Journ. Roy. Mic. Soc.' (N.S.). iv (1884), p. 317.

CHAPTER XXV.

CLEARING AGENTS.

277. Clearing agents are liquids whose primary function it is to make microscopic preparations transparent by penetrating amongst the highly refracting elements of which the tissues are composed, the clearing liquids themselves having an index of refraction not greatly inferior to that of the tissues to be cleared. Hence, all clearing agents are liquids of high index of refraction. The same substances have generally a second function which consists in getting rid of the alcohol in which preparations are generally preserved and facilitating the penetration of the balsam or other resinous medium in which preparations are, in most cases, finally mounted. Hence, all of the group of bodies here called "clearing agents," must be capable of expelling alcohol from tissues and must be at the same time solvents of Canada balsam and the other resinous mounting media.

It is important to note the manner of employing these agents. The old plan was to take the object out of the alcohol and float it on the surface of the clearing medium in a watch-glass. This plan was faulty, because the alcohol escapes from the surface of the object into the air quicker (in most instances) than the clearing agent can get into it; hence the object must shrink. To avoid or lessen this cause of shrinkage, clearing is now generally done by the method suggested by Giesbrecht, which consists in putting the clearing medium *under* the alcohol containing the object. This is done in the following manner. Take a test-tube, and put into it enough alcohol to contain the objects (— a watch-glass will often do well, but a test-tube is safer). With a pipette carefully put under the alcohol a sufficient quantity of clearing medium. Then put the objects into the alcohol. They will sink down to the level of separation of the two liquids at once; and after some time they will be found to have sunk to the bottom of the clearing medium. They may then be removed by means of a pipette; or the supernatant alcohol drawn off and the preparations allowed to remain until wanted.

The chief clearing agents are essential oils. A classification of these is given below (No. 279 Stieda, and No. 285 Schiefferdecker). Two very useful clearing media will be found in kreasote and carbolic acid, especially the latter, which is the most rapid of all known to me. These cause less shrinkage than the essential oils, and are easier to manage; but they should, as a general rule, not be employed for the purpose of preparing objects for balsam, as the preparations shrink by exosmosis after they have been a short time in balsam.

The penetration of all clearing media may be hastened by using them warm. Directions for clearing are given when necessary under the heads of the different organs and tissues. It will suffice here to advise the beginner to keep on his table the following: carbolic acid (or kreasote); clove oil; bergamot oil.

278. Absolute Alcohol as a Clearing Medium.—Absolute alcohol is recommended by Seiler for preparing objects for mounting in balsam, the balsam being in this case dissolved in warm absolute alcohol (*see* No. 352). The method is said to give very good results, but it is obvious that it is only applicable to cases in which it is not desired to make a preliminary examination of the cleared objects (for the sake of selecting the best or the like) before mounting them.

279. Kreasote as a Clearing Medium (*Stieda's method*).—

Stieda relates that kreasote was suggested him by a paper of Kutschin, "Über den Bau des Rückenmarks des Neunanges," Kasan, 1863. Kutschin rinsed his sections *in water*, brought them on to slides, drew off the water by means of blotting-paper, and added a drop of kreasote at the side. When clear, he covered, and closed the mounts with a border of dammar.

Stieda modified this process by mounting in dammar instead of kreasote.

He then tried experiments to ascertain whether oil of cloves could be applied in the same manner, that is, to the clearing of non-dehydrated sections. He found that it could, though its employment requires longer time. Sections brought from water into kreasote clear in a few minutes, whilst in oil of cloves they require from half an hour to an hour or more; and this slowness of the process exposes them to the risk of shrinkage.

Further experiments with other essential oils led him to establish the following classification:

A. The *turpentine* group, capable of clearing in a short time perfectly dehydrated sections, but clearing watery sections only after many hours or not at all.

Ol. Terebinthinæ.
Ol. Absynthii.
Ol. Balsam. Copaivæ.
Ol. Cortic. Aurantiorum.
Ol. Cubebarum.
Ol. Fœniculi.
Ol. Millefolii florum.
Ol. Sassafras.
Ol. Juniperi.
Ol. Menthæ cripsæ.
Ol. Origani vulgaris.
Ol. Lavandulæ.
Ol. Cumini.

Ol. Cajeputi.
Ol. Cascarillæ cortic.
Ol. Sabinæ.
Ol. Citri.

This, then, for Stieda, is the *Index Expurgatorius* of clearing media.

B. The *oil-of-cloves* group, clearing *very rapidly* sections that have been dehydrated, and clearing watery sections "somewhat more slowly" and with a certain amount of shrinkage.

Ol. Gaultheriæ.
Ol. Cassiæ.
Ol. Cinnamomi.
Ol. Anisi stellati.
Ol. Bergamotti.
Ol. Cardamomi.
Ol. Coriandri.
Ol. Carui.
Ol. Roris marini.

But Stieda remains convinced that kreasote is to be preferred to all other known clearing agents.

280. Carbolic Acid as a Clearing Medium.—Best used in concentrated solution in alcohol. Clears instantaneously, even very watery preparations. As remarked above, it is generally better avoided for preparations of soft parts which it is intended to mount in balsam, as they generally shrink by exosmosis when placed in the latter medium.

281. Xylol, Benzol, Chloroform.—All three are good for preparing objects for balsam, but are not useful as preliminary examination media on account of their great volatility.

282. Turpentine.—Generally used for treating sections that have been cut in paraffin, as it has the property of dissolving out the paraffin and clearing the sections at the same time. If used for alcohol objects it causes considerable shrinkage unless used in the thickened state, a method which is much liked for some purposes in Germany. Thickened turpentine ("Verhartzes Terpentinöl" of German writers) is prepared by exposing rectified turpentine in thin layers for some days to the air. All that is necessary is to pour some turpentine into a plate, cover it lightly so as to protect it from dust without excluding the air, and leave it until it has attained a syrupy consistency. Turpentine has, I believe, the lowest index of refraction of all the usual clearing agents; it clears objects *less* than balsam.

283. Oil of Cloves (*Rindfleisch's method*[1]).—The first advantage possessed by oil of cloves over turpentine is that it is *miscible* with strong alcohol. It is also miscible in all proportions with Canada balsam. The shrinkage inseparable from the use of turpentine is thus avoided on either hand, and the introduction of this medium is probably the first important step in the evolution of Lockhardt Clarke's original method.

Two important properties of clove oil should be noticed here. It does not easily spread itself over the surface of a slide, but has a tendency to form very convex drops. This property makes it a very convenient medium for making minute dissections in. The second property I wish to call attention to is that of making tissues that have lain in it for some time very brittle. This brittleness is also sometimes very helpful in minute dissections.

These qualities may be counteracted if desired by mixing the clove oil with bergamot oil.

Clove oil has, I fancy, the highest index of refraction of all the usual clearing agents; it clears objects *more* than balsam. It dissolves celloidin (or collodion) and therefore should not be used for clearing sections cut in that medium, without special precautions. Notwithstanding the opinion of Schiefferdecker, I consider this to be one of the best of clearing agents, and very valuable on account of the properties to which attention has been called above. I think no work-table should be without it.

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[1] 'Arch. Mik. Anat.,' i (1865), p. 138.

Clove oil darkens with age. Dark samples should be rejected, but so should *very* colourless ones, as these are generally adulterated. The proper colour is a pale sherry.

284. Oil of Bergamot.[1]—Schiefferdecker finds that this oil has many good qualities; it clears 95 per cent. alcohol preparations and celloidin preparations quickly, does not attack anilin colours, but the strong odour is disagreeable, it is as dear as oil of cloves, twice as dear as oil of origanum, and three times as dear as oil of cedar; he considers its action preferable to that of oil of cloves, but, all things considered, gives the palm to cedar and origanum. I think that this is a very valuable medium, and though I do not agree with Schiefferdecker in thinking its action superior to oil of cloves, I think it should always be kept at hand.

285. Ethereal Oils (*Neelsen and Schiefferdecker's experiments*[2]).—The authors examined a large series of ethereal oils (prepared by Schimmel and Comp., Leipzig), with the object of finding a not too expensive substance that should combine the properties of clearing quickly alcohol preparations, *not* dissolving out anilin colours, clearing celloidin without dissolving it, not evaporating too quickly, and not having a too disagreeable smell.

The following is a list of twenty-four products examined by them. It seems worth while to give it, although the authors only found three amongst the number that fulfil the conditions; as to know that they have been found wanting in some of these respects may perhaps save somebody a wild-goose chase.

Oils of—Anise, Amber, Birch-tar, Cajeput, Calmus, Cassia, Cedar wood, Citrons, Dill, Field-thyme, Firneedles, Mint, Cumin, Niobe, Origanum, Palmarosa, Peppermint, *Mentha*

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[1] 'Arch. Anat. u. Phys.,' 1882 (Anat. Abth.), p. 206.

[2] Ibid., p. 204.

Pulegium, Rosemary, Sassafras, Spikenard, Thuja, Sandalwood, Caraway.

Of these, the following three fulfil the conditions and can be *recommended*:—*Cedar wood, Origanum, Sandal wood.*

286. Cedar Oil[1].—Finest cedar-wood oil, price per kilo 4.20 mark (=4*s.* 2*d.*). Very thin, colour light yellow, odour slight (of cedar wood), evaporates slowly, is not changed by light, is miscible with chloroform-balsam and with castor-oil. Clears readily tissues in 95 per cent. alcohol, without shrinkage, does not extract anilin colours. Celloidin sections are cleared in five to six hours.

Cheap, but requires an inconvenient length of time for the clearing of celloidin sections.

Note.—I have examined the clearing properties of a sample of cedar-wood oil obtained from the celebrated firm of Rousseau, Paris. This sample was absolutely colourless. It *totally* failed to clear absolute alcohol objects after many days.

Cedar oil has, especially when thickened by exposure in thin layers to air and light, approximately the same refraction and dispersion as crown-glass. It is the most widely used immersion fluid for homogeneous immersion objectives. It is therefore probably the best *fluid* for examining objects with homogeneous lenses, if it be wished to make such an examination previous to mounting in balsam; I mean, the best fluid so far as regards the definition of such objectives, which are often unprovided with cover-corrections.

287. Oil of Origanum[2] (or "Spanishes Hopfen-Oel," price per kilo 15 mark (= 15*s.*). Thin, light-brown colour, odour not too strong, agreeable, does not evaporate too quickly, is not changed by light, is miscible with chloroform-balsam and with castor-oil. Ninety-five per cent. alcohol preparations are cleared quickly, and so are celloidin sections, without solution of the celloidin. Anilin colours are somewhat extracted.

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[1] Ibid.

[2] Ibid.

The authors think that a laboratory supplied with these two oils is fully equipped for all possible cases (the origanum oil being used merely to take the place of cedar-wood oil for the special case of celloidin sections).

288. Sandal-wood Oil[1].—"Finest East Indian sandal-wood oil," price per kilo 50 mark (= £2 10*s.* 0*d.*). Somewhat thicker than the last two, light yellow, odour faint, agreeable, evaporation hardly perceptible, unchangeable by light, miscible with chloroform-balsam and with castor-oil. Ninety-five per cent. alcohol preparations cleared quickly, celloidin more slowly, anilin colours unaffected.

Very useful, its worst fault is its high price.

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[1] Ibid.

CHAPTER XXVI.

INDIFFERENT LIQUIDS, EXAMINATION AND PRESERVATION MEDIA.

289. I comprehend under this heading all the media in which an object may be examined. The old distinction of "indifferent" liquids, and those which have some action on tissues appears to be misleading more than helpful; inasmuch as it is now well understood that *no* medium is without action on tissues except the plasma with which they are surrounded during the life of the organism; and this plasma itself is only "indifferent" whilst all is *in sitû;* as soon as a portion of tissue is dissected out and transferred to a slide in a portion of plasma the conditions become evidently artificial. It appears more useful here to group roughly the various media employed for microscopical examination according to the proportion of *water* they contain; the *aqueous* media which, abstracting little or no water from the tissues leave them so far in a relatively normal state, will include the traditional indifferent liquids; the absolutely waterless resins and essential oils will occupy the other end of the series; and partially dehydratant media (such as glycerin and alcohol) will occupy an intermediate position. This classification recommends itself from an optical point of view; since the series thus obtained represents very fairly that which would be obtained by grouping the media according to their indices of refraction, beginning with water, the liquid of lowest refraction known to us, passing through the somewhat more refractive albumens and syrups, through glycerin to the resins, and the still more highly refractive essential oils and other recently introduced mounting media.

It does not appear necessary to create a separate group for mounting media, as all preservative media may be used for mounting.

290. Water.—Water should always be distilled. To preserve it from mould, a lump of thymol or camphor should be kept in the supply. Water may be employed without inconvenience, and sometimes (on account of its low index of refraction, with great advantage) for the examination of all structures that have been fixed with osmic or chromic acid, or some salt of the heavy metals; but it is by no means applicable to the examination of fresh tissues, that is, tissues that have not been so fixed. It is important that the beginner should bear in mind that water is very far from being an "indifferent" liquid; many tissue elements are greatly changed by it (nerve-end structures for instance), and some are totally destroyed by its action if prolonged (for instance, red blood-corpuscles).

In order to render water inoffensive to such tissues as these it must, firstly, have dissolved in it some substance that will give it a density equal to that of the liquids of the tissue, so as to prevent the occurrence of osmosis, to which process the destructive action of pure water is mainly due. Salt solution is a medium suggested by this necessity. But salt solution by no means fulfils all the conditions implied in the notion of an "indifferent" liquid. In so far as it possesses a density approaching to that of the liquids of the tissues, one cause of osmosis is eliminated; but there remains another, due to the difference of composition of the liquids within the tissues and that without. Cell contents are a mixture of colloids and crystalloids, salt solution contains only a crystalloid, whose high diffusibility causes it to diffuse over into the colloids of the tissues. In order to reduce the consequent osmotic processes to a minimum, it is necessary that the examination medium contain in addition to a due proportion of salt or other crystalloid, also a due proportion of colloids. By adding for instance, white of egg to salt solution, this end may be attained, and as a matter of fact, the liquids recommended as indifferent are found invariably to contain both cystalloids and colloids. Thus (as stated by Frey) vitreous humour contains 987 parts of water to about 4·6 of colloid matters and 7·8 of crystalloids (common salt). In 1000 parts of the juice of fruits are contained about 3·8 parts of colloid matter (albumen), 5·8 of salt, and 3·4 of urea. In blood serum, 8·5 of colloids and 1 of crystalloid substance are found.

291. Salt Solution.—("Normal salt solution," "physiological salt solution") 0·75 per cent sodium chloride in water. Carnoy recommends the addition of a trace of osmic acid.

Serum.—(*See* **Iodised Serum, No. 447.**)

292. Artificial Iodised Serum (*Frey's formula*).

White of egg	30·0	grammes.
Sodium chloride	00·4	,,
Water	270·00	,,

Sufficient iodine may be added to make the mixture keep.

292a. Aqueous Humour, Fruit Juice, Simple White of Egg.—Require no preparation beyond filtering. They may be iodised if desired.

293. Syrup.—An excellent medium for examining many structures in the fresh state. To preserve it from mould, chloral hydrate may conveniently be dissolved in it (1 to 5 per cent.).

Chloralised Syrup.—1 per cent. of chloral is sufficient to preserve the syrup, but a larger proportion may be employed without hurt. I have used as much as 7 per cent. and found no disadvantage.

294. Carbolised Syrup.—Carbolic acid may be employed instead of chloral; 1 per cent. is sufficient.

Either of these syrups may be used as a mounting medium, but they are not to be recommended for that purpose, as there is always risk of the sugar crystallising out.

294a. Saliva.—Saliva has been recommended with the idea of its being innocuous to delicate structures; it is of course a macerating agent. (*See* MACERATING AGENTS, **Artificial Saliva**, No. 462.)

295. Carbolic Acid.—1 per cent. in water. Is a mounting medium.

296. Kreasote.—The 'Micrographic Dictionary' recommends kreasote water, "prepared by filtering a saturated solution of kreasote in rectified spirit mixed with 20 parts of water. It is recommended for preserving preparations of muscle, cellular tissue, tendon, cartilage, &c."

297. Kreasote Fluid (*Thwaites' fluid*[1]).

Water	16 ounces.
Spirits of wine	1 ,,

Kreasote sufficient to saturate the spirit.

Chalk as much as may be necessary.

Mix the kreasote and spirit, stir in the chalk with the aid of a pestle and mortar, and let the water be added gradually. Next add an equal quantity of water saturated with camphor. Allow the mixture to stand for a few days and filter.

This is a preservative solution. Beale found that it always became turbid if used for the preservation of large specimens, and was therefore led to the following modification of it:

298. Solution of Naphtha and Kreasote (*Beale's fluid*[2]).

Kreasote	3 drachms.
Wood-naphtha	6 ounces.
Water	64 ,,

Chalk as much as may be necessary.

First mix the naphtha and kreasote, then add as much prepared chalk as may be sufficient to form a thick smooth paste, afterwards add very gradually a small quantity of the water, which must be well mixed with the other ingredients in a mortar. Add two or three small lumps of camphor and allow the mixture to stand in a lightly-covered vessel for a fortnight or three weeks with occasional stirring. The almost clear supernatant fluid may then be poured off and filtered if necessary. It should be kept in well-corked or stoppered bottles.

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[1] Beale, 'How to Work,' &c., p. 55.

[2] Ibid., p. 56.

299. Wood-Naphtha Solution (*Quekett's fluid*[1]).—One part of wood-naphtha or pyro-acetic spirit to 10 of water. Filter if it becomes cloudy after standing.

300. Alum Sea-Water.—A saturated solution of alum in sea-water is useful for the examination and preservation of the tissues of many marine organisms (Medusæ, Siphonophora, Ctenophora, Pelagic Tunicata). The animals may be killed in the fluid, which is a fair fixing agent.

301. Acetate of Alumina (*Gannal's solution*[2]).

Acetate of alumina	1 part.
Water	10 „

302. Arsenious Acid Solution.[3]—Made by boiling excess of the acid with water, filtering the solution, and adding 2 parts of water.

303. Arsenite of Potash.[4]—"One part dissolved in 160 of water has been found useful for preserving the primitive nerve-tubes."

304. Carbonate of Potash.[5]—"One part dissolved in from 200 to 500 of water is a good preservative of the primitive nerve-fibres."

305. Acetate of Potash (*Max Schultze's formula*[6]).—A nearly saturated solution in water. It is used by letting a drop run in under the cover-glass to the object, which is in water. After twenty-four hours the mount may be closed. The index of refraction is lower than that of glycerin.

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[1] Beale, 'How to Work, &c.,' p. 56.

[2] Ibid., p. 58.

[3] 'Micro. Dict.,' Art. "Preservation," p. 640.

[4] Ibid.

[5] Ibid.

[6] 'Arch. Mik. Anat.,' vii (1872), p. 180.

306. Calcium Chloride[1].—Either about 1 part of the salt to 2 of water, or a saturated solution may be used. A lump of camphor should be added to the solution to preserve it. As this salt is very hygroscopic, its solution presents the advantage of not drying up, so that it is not necessary to close the mounts until it is desired to put them away.

307. Chloral Hydrate (*Lavdowsky's formula*[2]).—Five per cent. solution in water.

308. Chloral Hydrate (*Brady's formula*[3]).

Chloral hydrate . . .	12 grains.
Camphor water . . .	1 fluid ounce.

Recommended for the preservation of *Copepoda*. Glycerin-jelly (containing very little glycerin) is recommended for mounting.

309. Chloral Hydrate (*Munson's formula*[4]).—"Five grains to an ounce of water is strong enough for all small objects."

310. Gum with Chloral Hydrate or Acetate of Potash (*Hoyer's formula*[5]).—A high 60 c.c. glass with a wide neck is filled two thirds full with gum-arabic (in pieces), and then *either* a solution of chloral (of several per cent.) containing 5—10 per cent. of glycerin, is added, *or* acetate of potash or ammonia. The gum with frequent shaking dissolves in a few days, and forms a syrupy fluid, which is slowly filtered for twenty-four hours. The clear filtered fluid will keep a long time, but if spores of fungi begin to develop a little chloral can be added and the fluid refiltered. The solution with chloral is for carmine or hæmatoxylin objects, that with acetate for anilin objects.

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[1] 'Micro. Dict.,' Art. "Calcium, chloride of."

[2] 'Arch. Mik. Anat.,' xiii (1876), p. 359.

[3] "Monograph of the Free and Semiparasitic Copepoda of the British Islands," 'Journ. Roy. Mic. Soc.' i (1878), p. 369.

[4] 'Amer. Journ. Micr.,' vi (1881), pp. 117-18. 'Journ. Roy. Mic. Soc.' (N.S.), i (1881), p. 847.

[5] 'Biol. Centralb.,' ii (1882), pp. 23-4. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), pp. 144-5.

311. Corrosive Sublimate Solution (*Harting's fluid*[1]).—One part of sublimate to from 200 to 500 of water. (For blood-corpuscles of frog 1—400, of birds 1—300, of mammals 1—200.) "Harting recommends this as the best preservative for the corpuscles of the blood, nerve, muscular fibre, &c."

Pacini[2] remarks that "bichloride of mercury coagulates and precipitates the albuminous matter that exists in the interstitial fluids of the tissues," and therefore in order to prevent this coagulation it is well to associate with it salt for certain preparations, or acetic acid for others. On this principle are prepared the following classical fluids of Goadby and Pacini.

312. Salt, Alum, and Corrosive Sublimate ("Goadby") (*Goadby's fluid*[3]).

Bay salt (coarse sea salt) . .	4 ounces.
Alum	2 "
Corrosive sublimate . . .	2 grains.
Boiling water	1 quart.

This is found to be "too strong" for most purposes, and therefore the following is recommended for general purposes.

313. Salt, Alum, and Corrosive Sublimate (*Goadby's second fluid*[4]).

Bay salt	4 ounces.
Alum	2 "
Corrosive sublimate . . .	4 grains.
Water	2 quarts.

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[1] 'Micro. Dict.,' Art. "Preservation," p. 640.

[2] 'Journ. de Mic.,' iv (1880). 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 702.

[3] 'Micro. Dict.,' Art. "Preservation," p. 641.

[4] Ibid.

"Schultze recommends it for preserving *Medusæ*, *Echinodermata*, Annelid larvæ, *Entomostraca*, *Polythalamia*, and *Polycystina*, and advises the use of glycerin afterwards to produce transparence."

314. Salt and Corrosive Sublimate (*Goadby's third fluid*[1]). When carbonate of lime exists in the preparations, the alum must be omitted. The following formula is recommended:

Bay salt	8 ounces.
Corrosive sublimate	2 grains.
Water	1 quart.

315. Salt and Corrosive Sublimate (*Goadby's fourth fluid*[2]). "Marine animals require a stronger fluid of this kind, made by adding about 2 ounces more salt to the last."

316. Salt and Corrosive Sublimate (*Pacini's fluid, No.* 2[3]).

Bichloride of mercury	1 part.
Common salt	2 ,,
Water	200 ,,

Of general employment, but especially useful for blood-corpuscles of cold-blooded animals, as it has a less density than the following fluid. It preserves spermatic fluid, epithelia, nerves, and muscle fibres. It is also used for fixing Infusoria, a small quantity being added to the water containing them.

317. Salt and Corrosive Sublimate (*Pacini's fluid, No.* 3[4]).

Bichloride of mercury	1 part.
Common salt	4 ,,
Water	200 ,,

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[1] Ibid.

[2] Ibid.

[3] Journ. de Mic.,' iv (1880). 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 702.

Note.—Pacini's fluid, No. 1, is identical with that of Harting given above, viz. 1·200 sublimate in water. Pacini uses it for removing, when desired, the salt or acid from preparations that have been placed in one of the other solutions.

[4] Ibid.

For blood-corpuscles of warm-blooded animals.

318. Acetic Acid and Corrosive Sublimate (*Pacini's fluid, No. 4*[1]).

Bichloride of mercury . . .	1 part.
Acetic acid	2 ,,
Water	300 ,,

"Serves best for the nuclei of animal tissues, but it swells up the fibres and distorts the forms of the cells."

319. Salt, Sublimate, and Glycerin (*Pacini's formula*[2]).

In the place here quoted, Frey speaks of the liquids of Pacini as differing from those of Goadby through their containing glycerin in lieu of alum. He gives the following directions. Take—

Sublimate	1 part.
Sodium chloride	2 ,,
Glycerin (25° Beaumé) . . .	13 ,,
Water	113 ,,

Allow the mixture to remain undisturbed for at least two months. At the end of that time, take for use 1 part, mix with 3 parts of water, and filter. This mixture is said to be a good preservative of all delicate tissues.

320. Acetic Acid, Sublimate, and Glycerin (*Pacini's formula*[3]):

Sublimate	1 part.
Acetic acid	2 ,,
Glycerin (25° Beaumé) . . .	43 ,,
Water	115 ,,

This mixture is to be employed in the same way as the last. It is said to destroy red blood-corpuscles, but to preserve white blood-corpuscles.

321. Modifications of the foregoing Sublimate Solutions.[4]—The following formulæ are quoted by Frey from Cornil as being in use in the Pathological Institute of Berlin.

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[1] Ibid.

[2] Frey, 'Le Microscope,' 1867, p. 233.

[3] Ibid.

[4] Ibid.

1.	Sublimate	1 part.
	Sodium chloride	2 „
	Water	100 „

For the more vascular tissues of warm-blooded animals.

2.	Sublimate	1 part.
	Sodium chloride	2 „
	Water	200 „

For similar tissues of cold-blooded animals.

3.	Sublimate	1 part.
	Sodium chloride	1 „
	Water	300 „

For pus-corpuscles and analogous elements.

4.	Sublimate	1 part.
	Water	300 „

For blood-corpuscles.

5.	Sublimate	1 part.
	Acetic acid	1 „
	Water	300 „

For epithelia, connective tissue, and pus-corpuscles, when it is desired to demonstrate the nuclei.

6.	Sublimate	1 part.
	Acetic acid	3 „
	Water	300 „

For ligaments, muscles, and nerves.

7.	Sublimate	1 part.
	Acetic acid	5 „
	Water	300 „

For glandular tissues.

8.	Sublimate	1 part.
	Phosphoric acid	1 „
	Water	30 „ (*sic.*)

For cartilaginous tissues.

322. Salicylic Vinegar Preservative Solutions (*Fr. Meyer's formula*[1]).—"Salicylic vinegar" is a solution of 1 part of salicylic acid in 100 parts of pyroligneous acid. The pyroligneous acid should be of 1·04 specific gravity, and should be of a pale yellow colour. This product is found in commerce and may be obtained from Herrn J. M. Andreœ, Droguerie-Handlung, Frankfurt a. M.

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[1] 'Arch. Mik. Anat.,' xiii (1876), p. 868.

First formula.

One vol. salicylic vinegar to 10 vols. of the following dilute glycerin : viz. glycerin 1 vol., water 2 vols.

For various Larvæ, Hydræ, Nematodes, &c.

Second formula.

One vol. salicylic vinegar to 10 vols. of the following dilute glycerin: viz. glycerin 1 vol., water 4 vols.

For Infusoria.

323. Salicylic Vinegar and Gum Medium (*Noll's method*[1]).—A mixture of equal vols. of Meyer's second fluid (*ante*, last formula) and Farrant's medium (*post*, No. 330).

This mixture never becomes turbid and does not dry up. The covers may be luted with asphalt or any other cement. The fluid answers admirably for delicate Crustaceæ and their larvæ, the preparations do not shrink, and are not too much cleared. It also answers well for hardened and stained preparations of Hydroids, small Medusæ, and other cœlenterates.

324. Alcoholic Sublimate Solution (*Gibson's formula*[2]) :

Alcohol of 60 per cent. . .	60 c.c.
Water	30 „
Glycerin	30 „
Acetic acid (15 parts of the glacial to 85 of water) . . .	2 „
Bichloride.	0·15 grammes.

325. Chloride and Acetate of Copper (*Ripart et Petit's formula*[3]) :

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[1] 'Zool. Anz.,' vi (1883), p. 472.

[2] Carnoy, 'La Biologie Cellulaire,' p. 94.

[3] Ibid.

Camphor water (not saturated)	75 grammes.
Distilled water . . .	75 „
Crystallised acetic acid . .	1 „
Acetate of copper . . .	0·30 „
Chloride of copper . .	0·30 „

Carnoy states that this is a most admirable examination medium. The most delicate elements are perfectly preserved in it; the addition of a drop of osmic acid or corrosive sublimate does not cause the least turbidity and enhances its *fixing* action.

326. **Tannin** (*Carnoy's formula* [1]):

Water	100 grammes.
Powdered tannin . . .	0·50 „

327. **Picro-Carmine.**—Picro-carmine has been recommended by Ranvier as a medium for teasing fresh tissues in, in the belief that it possesses sufficient fixing action to preserve the forms of cells. Carnoy finds that cells live in it for a considerable time, and become gorged with water and deteriorated to a considerable degree. Unfortunately, too, picro-carmine cannot be combined with a good fixing agent, as it is precipitated by alcohol and by acids, and especially by osmic acid.

328. **Methyl-Green.**—See under STAINING AGENTS. The aqueous solution is strongly recommended by Carnoy as an examination medium for fresh tissues. It should be taken fairly concentrated, in which state it has sufficient fixing power, which is enhanced by the addition of a trace of osmic acid.

329. **Gelatin and Honey Medium** (*Deane's medium* [2]):

Gelatin	1 ounce.
Honey	5 „
Water	5 „
Rectified spirit	½ „
Kreasote	6 drops.

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[1] Ibid., p. 95.

[2] 'Micro. Dict.,' Art. "Preservation."

The gelatin is soaked in the water till soft and then added to the honey, which has previously been raised to a boiling heat in another vessel; then boil the mixture, and when it has cooled somewhat add the kreasote mixed with the spirit; lastly, filter through fine flannel. The medium must be warmed and a warm slide employed for mounting.

330. Gum and Glycerin Medium (*Farrant's medium*[1]):

Picked gum-arabic	4 ounces.
Water	4 „
Glycerin	2 „

To be kept in a stoppered bottle with a lump of camphor.

This medium is quoted by Frey as consisting of equal parts of gum, glycerin, and saturated aqueous solution of arsenious acid.

The 'Micrographic Dictionary' gives the following directions:

Gum-arabic 1 ounce, glycerin 1 ounce, water 1 ounce, arsenious acid 1½ grains; dissolve the arsenious acid in the water, then the gum (without heat), add the glycerin, and incorporate with great care to avoid forming bubbles.

331. Gum and Glycerin Medium (*Langerhans' formula, modification of Farrant's medium*[2]).

Gummi arab.	5·0.
Aquæ	„

To which after twelve hours are added—

Glycerini	5·0.
Sol. aquosa acid. carbol. (5·100)	10·0.

Marine animals may be preserved in this by simply running in a drop under the cover, and next day or later adding what is necessary to make up for evaporation, and closing the mount. Shrinkage is very slight, and most colours keep well.

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[1] Beale, 'How to Work, &c.,' p. 58.

[2] 'Zool. Anzeig,' ii (1879), p. 575.

332. Gum and Syrup Medium (*Cole's formula*[1]).—Gum mucilage (B. P.) 5 parts, syrup 3 parts. Add 5 grains of pure carbolic acid to each ounce of the medium. (For brain, spinal cord, and retina, and all tissues liable to come in pieces, put 4 parts of syrup to 5 of gum.)

(Gum mucilage (B. P.) is made by placing 4 ounces of picked gum acacia in 6 ounces distilled water, stirring occasionally until the gum is dissolved. This is to be strained through muslin. The syrup is made by dissolving 1 pound of loaf sugar in 1 pint of distilled water and boiling.) This medium is intended for preserving tissues to be cut with the freezing microtome, and for freezing in; it is not a mounting medium.

333. Glycerin-Gelatin ("Glycerin-jelly") (*Deane's formula*[2]). —120 grammes glycerin, 60 grammes water, 30 grammes gelatin. Dissolve the gelatin in the water, and add the glycerin. This, and the following glycerin-jellies, must of course be used warm.

334. Glycerin-Gelatin ("Glycerin-jelly") (*Lawrence's formula*[3]).—"He takes a quantity of Nelson's gelatin, soaks it for two or three hours in cold water, pours off the superfluous water, and heats the soaked gelatin until melted. To each fluid ounce of the gelatin, *whilst it is fluid but cool,* he adds a fluid drachm of the white of an egg. He then boils this until the albumen coagulates and the gelatin is quite clear, when it is to be filtered through fine flannel, and to each ounce of the clarified solution adds 6 drachms of a mixture composed of 1 part of glycerin to two parts of camphor-water."

335. Glycerin-Gelatin (*Beale's formula*[4]).—Gelatin or isinglass, soaked, melted, and clarified if desired, as in the last formula. To the clear solution add an equal bulk of strong glycerin.

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[1] 'Methods of Microscopical Research,' 1884, p. xxxix. 'Journ. Roy. Mic. Soc.' (N.S.), iv (1884), p. 318.

[2] Frey, 'Le Microscope,' p. 231.

[3] Davies, 'Preparation and Mounting of Microscopic Objects,' p. 84.

[4] 'How to Work, &c.,' p. 57.

336. Glycerin-Gelatin (*Brandt's method of preparation*[1]). —Melted gelatin 1 part, glycerin 1½ parts.

The gelatin to be soaked in water and melted in the usual way. After incorporating the glycerin, the mixture is to be filtered. This is a point of vital importance, as the gelatin of commerce is always mixed with particles of dust and minute threads. Swedish filtering-paper does not allow the fluid to pass through sufficiently, and flannel produces more threads than before. The following simple apparatus is found effective. A wide-necked bottle is broken in two, and the upper part taken. The neck is stopped with a cork having two holes bored in it. In the first hole a glass tube about 20 cc. long is inserted so as to project a little into the inside of the bottle and on the outside it is bent sharply to one side and drawn out into a point of about 1½ to 2 mm. diameter. In the second hole a funnel-shaped filter is inserted so that the conical part is inside the bottle and the tube projects a few centimetres beyond the cork and the neck of the bottle. The apparatus is then placed so that the wide opening of the bottle and of the funnel is uppermost, and some spun glass is pressed into the lower conical part of the filter. In using the apparatus, the funnel is filled with glycerin-gelatin, and the bottle with hot water, which runs off slowly through the tube in the first hole and is constantly replenished.

Some drops of carbolic acid should be added to the fluid product of the filtering. For mounting, use warm, by melting a small portion on the slide, the object having been previously soaked for some time in a small bottle of the medium warmed with a suitable apparatus.

337. Glycerin-Gelatin (*Kaiser's formula*[1]).—One part by weight finest French gelatin is left for two hours in 6 parts by weight distilled water, 7 parts of glycerin are added, and for every 100 grammes of the mixture 1 gramme of concentrated carbolic acid. Warm for ten to fifteen minutes, stirring all the while, until the whole of the flakes produced by the carbolic acid have disappeared. Filter whilst warm through the finest spun glass laid wet in the filter. Use for mounting as above.

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[1] 'Zeitschr. f. Mik.,' ii (1880), p. 69. 'Journ. Roy. Mic. Soc.' iii (1880), p. 502.

[2] 'Bot. Cent.,' i (1880), p. 25. 'Journ. Roy. Mic. Soc.,' iii (1880), p. 504.

I prepared some of this jelly three years ago, and find it is still perfectly clear.

338. Glycerin-Jelly (Isinglass) (*Seaman's formula*[1]).—Dissolve isinglass in water, so that it makes a stiff jelly when at the ordinary temperature of the room, add one tenth as much glycerin, and a little solution of borax, carbolic acid, or camphor water. Filter whilst warm through muslin, and add a little alcohol.

339. Wickersheimer (*Wickersheimer's first formula*[2]).—In 3000 grammes boiling water dissolve 100 grammes alum, 25 grammes common salt, 12 grammes saltpetre, 60 grammes potash, and 20 grammes arsenious acid.[3] Allow the solution to cool and filter. To 10 litres of the neutral, colourless, and inodorous solution add 4 litres of glycerin and 1 litre methylic alcohol.

This liquid was at first stated to be applicable to the preservation of all kinds of anatomical specimens, but later experiments convinced Wickersheimer "that one and the same mixture is not suited for all objects; and he has therefore made four different kinds." No. 3 of these is for microscopical purposes. I have not been able to discover the formula, cf. 'Entomol. Nachr.,' vi (1880), p. 129, quoted in 'Journ. Roy. Mic. Soc.,' iii (1880), p. 855.

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[1] 'Amer. Mon. Mic. Journ.,' ii (1881), pp. 4-5. 'Journ. Roy. Mic. Soc.' (N.S.), i (1881), p. 534.

[2] 'Zool. Anz.,' 1879, p. 670.

[3] In the specification of the patent 10 grammes arsenious acid are given, by a printer's error, instead of 20. Conf. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 427.

340. Boroglyceride (*Barff's formula*[1]).—Boracic acid is dissolved in glycerin by the aid of heat, the solution taking about four or five hours, care being taken that the temperature employed be not so excessive as to decompose the glycerin. To the solution or compound further quantities of boracic acid are added from time to time until the boracic acid ceases to be dissolved. The compound resulting when allowed to cool is solid, and is called by the patentee boroglyceride.

In order to employ the compound, a solution is prepared in water, alcohol, or other suitable solvent. Barff finds that a solution of about 1 part by weight of the compound and 40 parts by weight of water, gives good results. The solutions are stated to be applicable to the preservation of all animal or vegetable substances.

341. Borax-Salicylic Acid Medium (*Mickle's formula*[2]).—Salicylic acid "dissolves very sparingly in water, and alcohol produces changes which are frequently undesirable. It is well known that salicylic acid dissolves freely in solution of borax, and it is also familiar to most persons that borax itself is quite efficient as a preservative. It therefore occurred to him to combine them, 2 parts of salicylic acid and 1 part of borax dissolving completely in half an ounce of glycerin" (*sic*), "this solution when mixed with 3 parts of water forming an excellent preservative fluid for coarse organisms. More delicate preparations should be mounted in the above solution diluted with 5 parts of water."

There is no danger of the salts crystallising out and spoiling the object.

342. Glycerin.—Glycerin diluted with water is frequently employed as an examination and mounting medium. Dilution with water is sometimes advisable from an optical point of view, on account of the increased visibility that it gives to many structures by lowering the index of refraction of the glycerin. But from the point of view of efficacious preservation, it is always advisable to use undiluted glycerin, the strongest that can be procured.

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[1] 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), pp. 124-5.

[2] 'Am. Journ. Mic.,' v (1880), pp. 185-6. 'Journ. Roy. Mic. Soc.,' iii (1880), pp. 1037-8.

Long soaking of tissues in glycerin of gradually increased strength is a necessary preliminary to mounting in all cases in which it is desired to obtain the best possible preparations and to ensure that they shall keep well. If this soaking is done on the slide (the cover being removed and the object treated with fresh glycerin every one or two days), it is well to take the precaution recommended by Beale, of luting the edges of the cover so as to make the preparation airtight, as glycerin is so highly hygroscopic that a drop of it exposed to the air rapidly diminishes in strength to a very considerable degree. In order to facilitate the removal of the cover in this process, the slide may be gently warmed by passing it two or three times through the flame of a spirit-lamp. No preparation can be considered to be made *secundum artem* until every part of the object has been thoroughly impregnated with strong pure glycerin.

The shrinking that frequently occurs when delicate structures are brought into glycerin may generally be cured by this treatment; cells which at first appear hopelessly collapsed gradually swell out to their normal forms and dimensions.

For closing glycerin mounts, the edges of the cover should first (after having been cleansed as far as possible from superfluous glycerin) be painted with a layer of *glycerin-jelly;* as soon as this is set a coat of any of the usual cements may be applied.

Glycerin dissolves carbonate of lime, and is therefore to be rejected in the preparation of calcareous structures that it is wished to preserve.

Glycerin does not preserve hæmatoxylin[1] or cochineal stains. For the preservation of carmine stains it should be acidulated with 1 per cent. of formic or acetic acid. Glycerin is useful for preserving (until wanted for section-cutting) tissues that have been treated with osmic acid, as it prevents them from becoming brittle as they otherwise do.

The already high index of refraction of glycerin (Price's glycerin, $n = 1·46$) may be raised to about that of crown-glass by dissolving suitable substances in the glycerin. Thus the refractive index of a solution of chloride of cadmium ($CdCl_2$)[2] in glycerin may be 1·504; that of a saturated solution of sulpho-carbolate of zinc in glycerin may be 1·501; that of a saturated solution of Schering's[3] chloral hydrate (in crusts) in glycerin is 1·510; that of iodate of zinc in glycerin may be brought up to 1·56.[4] The clearing action of glycerin may thus be greatly increased, and the full aperture of homogeneous objectives brought to bear on objects mounted in one of the above-named solutions.

The sulpho-carbolate of zinc solution[5] may be prepared by taking equal parts by weight of Price's glycerin and sulpho-carbolate of zinc crystals, mingling the two, and applying sufficient heat to boil the glycerin. The solution can be made in about an hour, but no fear need be had about boiling too long, as the longer this is done the less liability will there be for the solution to deposit crystals on the bottom of the bottle when cooled, which it will do if the temperature is only kept up long enough to dissolve the crystals. Filter while hot. The index may be brought up to

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[1] It is generally believed that glycerin is an efficient preservative for hæmatoxylin preparations, but I consider that it cannot be relied on to keep the stain perfect.

[2] 'Journ. Roy. Mic. Soc.,' ii (1879), p. 346.

[3] Ibid (N.S.), i (1881), p. 943.

[4] Ibid., p. 366.

[5] Ibid., iii (1880), p. 1051.

1·525 if desired, by evaporating the solution somewhat, or by adding more carbolate.

343. Glycerin and Alcohol Mixture (*Häntsch's fluid*[1]).

Alcohol	3 parts.
Glycerin	1 ,,
Water	2 ,,

The proportions of this mixture may frequently be varied with advantage. I have found the following very useful:

344.

Alcohol	1 part.
Glycerin	1 ,,
Water	1 ,,

as well as fluids weaker in alcohol and glycerin, down to—

345.

Alcohol	1 part.
Glycerin	1 ,,
Water	3 ,,

346. Dilute Alcohol (*Carpenter's formula*[2]).

Alcohol	1 part
Water	5 ,,

347. Biniodide of Mercury and Iodide of Potassium (*Stephenson's formula*[3]).—A solution of the two salts in water. "This is very easily prepared by adding the two salts to the water until each shall be in excess; when this point of saturation has been reached the liquid will be found to have a refractive index of 1·68, by far the highest aqueous solution known to me. Its advantages from an optical point of view are considerable, and it may be used of any strength; commencing with pure water, with a refractive index of 1·33, we can go on progressively to 1·465, which represents glycerin, still on to 1·54 (Canada balsam), again onwards to 1·624, which represents bisulphide of carbon, to 1·658, which represents the monobromide of naphthalin, to 1·662, the equivalent of a solution of sulphur in bisulphide of carbon, until, undiluted, it finally reaches its own maximum of 1·680; thus we have the representatives of all these media and an infinite number of others in this one fluid."

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[1] 'Micro. Dict.,' Art. "Preservation."

[2] 'The Microscope,' p. 246.

[3] 'Journ. Roy. Mic. Soc.,' (N.S.), ii (1882), p. 167.

This fluid is very dense, its specific gravity being 3·02. It is highly antiseptic.

"Its refractive index being 1·68, the visibility of diatoms, when mounted in it, is represented by the number 25 as compared wlth 11 in Canada balsam, in other words, the image is nearly two and a half times as strong. . . . . . For muscular fibre, on the other hand, a strong solution is not suitable, since the high refractive power of the object approaches that of the medium, but as every other medium, of a lower index than 1·68 can, by dilution, be represented by it, any degree of visibility down to that of water can be obtained.

"For marine animals a weak solution is probably well adapted, as about a 1 per cent. solution (5 minims to the ounce) will give the specific gravity of sea-water, with no appreciable difference in the refractive index."

Covers should be sealed with white wax, and the mounts finished with two or three coatings of gold-size and one of shellac.

348. Canada Balsam.—Canada balsam should always be used cold, in solution (unless it is desired to demonstrate the lacunæ of bone, or similar structures, as air-spaces). The solvents generally employed are xylol (or benzol), chloroform, and turpentine; absolute alcohol is also used, but seldom. It is well, in all cases, to evaporate the balsam at a gentle heat until it becomes brittle when cool, before adding the menstruum.

349. Xylol or Benzol-Balsam.—Equal volumes of balsam and xylol or benzol. Xylol is to be preferred. (This mixture suffices to clear paraffin-sections, if not too thick.)

350. Chloroform-Balsam.—Dissolve balsam in sufficient chloroform to give the required consistency. This, as well as benzol or xylol balsam, sets quickly.

Heys states that if the solution be poured into long, thin, half-ounce phials, corked up, and set aside for at least a month, the medium will be clearer and set much quicker than if the balsam is mixed with the chloroform at the time it is required for use.[1]

351. Turpentine-Balsam.—Dissolve balsam in sufficient turpentine to give the required consistency. This medium sets very slowly, which is in many cases an advantage, as it allows the operator to take time with the arrangement of his object. Seldom used, and, I think, unjustly neglected. Should be recommended to the beginner in preference to the preceding formulæ. Beale states that turpentine-balsam is apt to become streaky some time after the preparation has been mounted, and that bubbles are often found in it.

352. Alcohol-Balsam (*Seiler's formula*[2]).—"Take a clear sample of Canada balsam and evaporate it in a water or sand-bath to dryness; *i.e.* until it becomes brittle and resinous when cold. Dissolve this while warm in warm absolute alcohol and filter through absorbent cotton."

The advantage of this medium is that objects may be mounted in it direct from absolute alcohol, without previous treatment with an essential oil or other clearing agent; Seiler considers that by this means "shrivelling is avoided, as well as *the solution of fat in the cells.*"

353. Colophonium.—A solution of colophonium in turpentine has been recommended by Kleinenberg. I find it works very pleasantly. The palest kinds of colophonium should of course be taken.

This medium sets very slowly, so that ample time is afforded for arranging objects in it. Kleinenberg warns against the employment of absolute alcohol as a solvent; the preparations are beautiful at first, but soon become spoiled by the precipitation of crystals or of an amorphous substance.

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[1] 'Trans. Mic. Soc.,' Jan., 1865, p. 19. Beale, p. 51.

[2] 'Proc. Amer. Soc. Mic.,' 1881, pp. 60-2. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), pp. 126-7.

354. Dammar (*Flemming's formula*[1]).—Dissolve gum dammar with the aid of heat in a mixture of equal parts of benzol and turpentine and evaporate to the consistency of a thick syrup.

This medium works very pleasantly, and gives, I think, for some reason or other, better preparations of delicate tissues than any other medium with which I am acquainted. But unfortunately it appears not to be stable. I have always found my solutions to become cloudy, and to contain a granular substance in suspension, after a few months. I have therefore reluctantly abandoned this medium and do not recommend it.

355. Dammar Varnish.[2]—" C. J. M." explains that dammar is not entirely soluble in ether (*sic*), benzol or turpentine at ordinary temperatures; whilst if heat be used the solution is more complete, but sooner or later the product will become milky, and then it will be found impossible to clarify it.

To obtain a perfectly limpid solution, permanently remaining so, proceed as follows:

To 4 drachms of crushed Indian dammar add 8 fluid drachms of pure benzol, and allow the resin to dissolve at the ordinary temperature. After a day or two, an insoluble residue will be found at the bottom of the vessel. Carefully decant the supernatant clear liquid, and add to it 80 minims ($1\frac{1}{3}$ drachm) of spirits of turpentine. The object of adding turpentine is to ensure toughness in the dried film.

356. Dammar Varnish (*Pfitzner's formula*[3]).—Equal parts of dammar, benzin, and turpentine, are put to dissolve in a warm place. As soon as complete solution has taken place the clear liquid is poured off, and allowed to evaporate to the required consistency. Max Flesch notes hereon ('Zool. Jahresber. f. 1880,' p. 51) that at Würzburg the ordinary dammar varnish of artists is employed.

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[1] 'Arch. Mik. Anat.,' xix (1881), p. 322.

[2] 'Sci. Gossip,' 1882, p. 257. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 145.

[3] 'Morphol. Jahrb.,' vi (1880), p. 469. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 583.

356 a. Cedar Oil and Dammar.[1]—A solution of gum dammar in hot oil of cedar-wood has been recommended as a homogeneous-immersion fluid by Abbe. It may very likely be found useful as a mounting medium. The cedar oil examined by Abbe had a refractive index of 1·51 only, but by the addition of dammar this can be raised to 1·54. This solution is rather highly coloured, but if it be carefully distilled it becomes sufficiently pale and loses its stickiness. Like pure cedar oil, it does not act upon sealing-wax varnish nor upon shellac varnish,

356 b. Xylol-Dammar.—I find that dammar is *perfectly* soluble in xylol, in the cold, but when dry is very brittle.

356 c. Chloroform-Dammar.—M. Duval states ('Précis de Technique Microscopique,' p. 250) that he has made parallel experiments with chloroform-balsam and chloroform-dammar, and has not found, after a considerable lapse of time, any reason for preferring one of the two media to the other.

356 d. Balsam-Dammar.[2]—Vogt and Jung find that a solution of dammar in turpentine presents no point of superiority to benzol-balsam except that it is colourless, and they find that it becomes brittle when dry. The best results are obtained by means of a mixture of equal volumes of benzol-balsam and turpentine-dammar, the dammar serving to lighten the colour of the balsam.

Essential Oils.—*See* CLEARING AGENTS, under which heading these media will be found sufficiently discussed.

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[1] 'Journ. Roy. Mic. Soc.' (N.S.), i (1881), p. 366.

[2] 'Traité d'anat. comp. pratique,' p. 31.

357. Gum Styrax (or **Storax**[1]).—Storax is a liquid balsam obtained from the bark of *Liquidambar orientale.* It is of the consistence of birdlime, almost opaque, with an aromatic odour, and of a brownish-yellow colour. When pure it is soluble in alcohol and ether. Heated in a test-tube on the water-bath it becomes more liquid, but should give off no moisture.

F. Kitton[2] writes that to prepare it for microscopical purposes the resin must be dissolved in one of the following menstrua: chloroform, benzol, ether, or a mixture of benzol and absolute alcohol. The solution should be filtered and is then ready for use. It should be of the colour of brown sherry and the consistency of limpid olive oil. "The whole of the benzol should be eliminated by evaporation before placing the cover-glass on the slip. Its refractive index is then 1·63, very nearly that of monobromide of naphthalin." (These directions apply of course to the mounting of diatoms.) Dr Van Heurck directs that the commercial gum styrax should be exposed in thin layers to the light and air for several weeks, to eliminate the moisture contained in it previous to dissolving it, but Kitton has not found this necessary.

The 'Journ. Roy. Mic. Soc.' for June, 1884, says that the styrax supplied by Allen and Hanbury has a refractive index of 1·585 very nearly. This gives, for diatoms, a marked increase of visibility over Canada balsam ($n = 1·52$), as if we take the index of diatomaceous silex to be 1·43, balsam gives a visibility of 9, and styrax more than 15.

A. C. Cole considers that styrax is a *perfect* substitute for balsam; the solution is even easier to work with, and may be considered absolutely permanent and unalterable.

Styrax is officinal in England (*Styrax præparatus, prepared*

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[1] Garrod, 'Materia Medica,' p. 353.

[2] 'Sci. Gossip' 1884, p. 66. 'Journ. Roy. Mic. Soc.' (N.S.), iv (1884), pp. 318-19.

storax). In the drug trade it is known as "strained gum styrax." Van Heurck,[1] who first suggested this medium, recommended styrax from *Liquidambar styraciflora* and liquidambar from *L. orientalis*. "Liquidambar is preferable as being very pale yellow instead of a brownish yellow, but it does not appear to be obtainable from European druggists." It is officinal in America. Van Heurck's styrax was supplied by Gebe and Co., of Dresden.

358. Monobromide of Naphthalin.[2]—This liquid is colourless and oleaginous, with the odour of naphthalin. It is soluble in alcohol and ether and has a density of 1·555, with a refractive index of 1·658, giving therefore as the "index of visibility" 22 as against 11 for Canada balsam. It is not volatile.

It was found by Abbe and Van Heurck that this medium gave good results with diatoms. Max Flesch[3] experimented with it for histological preparations, and found that though in many cases the results did not bear out his expectations, yet there is probability that some objects will be better shown in it than in other media. When first mounted, preparations are very sharply defined, and it appears certain that they give better images than Canada balsam or glycerin preparations. After the lapse of a year and a half the preparations are found to be well preserved, but are no longer superior to Canada balsam preparations.

Tissues must be very carefully dehydrated before mounting as the least trace of moisture causes turbidity; they may either be brought direct from absolute alcohol into the medium or be first cleared with turpentine (clove oil and kreasote do not answer so well). The mounts may be luted with wax followed by shellac-varnish, or with thickened "Venetian turpentine." Dippel[1] recommends wax followed by "a cement of isinglass dissolved in spirit (called Heller's porcelain cement), or Canada balsam, rather thick, dissolved in chloroform, finally closing with a layer of shellac.

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[1] 'Bull. Soc. Belg. Mic.,' ix (1883), pp. 134-6. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 741.

[2] 'Journ. Roy. Mic. Soc.,' iii (1880), p. 1043.

[3] 'Zool. Anz.,' v (1882), p. 555.

[1] Journn. Roy. Mic. Soc.,' l. c.

CHAPTER XXVII.

CEMENTS AND VARNISHES.

359. Thanks to the efforts of the dilettanti to outshine one another with neatly gaudy "rings," microscopical literature contains a goodly show of receipts for cements and varnishes. I have collected such as appear likely to be useful, rejecting all that relates merely to ornament.

Two, or at most three, of the media given below, will certainly be found sufficient for all useful purposes. For many years I have used only one cement (Bell's). I recommend this as a cement and varnish; gold-size may be found useful for turning cells; and Ziegler's white cement or zinc white may be kept for occasions on which the utmost solidity is required.

Marine glue is necessary for making glass cells.

Carpenter lays great stress on the principle that the cements or varnishes used for fluid mounts should always be such as contain *no mixture of solid particles;* he has always found that those that do, although they might stand well for a few weeks or months, yet always became porous after a greater lapse of time, allowing the evaporation of the liquid and the admission of air. All fluid mounts *should be ringed with glycerin-jelly before applying a cement; by this means all danger of running-in is done away with.*

360. Asphalt Varnish.[1]—The 'Micrographic Dictionary' says this varnish consists of a solution of asphalt in boiling linseed oil, or oil of turpentine, or in a mixture of the two.

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[1] ('Bitume de Judée.')

It can be procured from the opticians or from the oilshops. Kitton ('Month. Mic. Journ.,' 1874, p. 34) recommends asphalt dissolved in benzol with the addition of a small quantity of gold-size.

361. Bell's Cement.—Composition unknown. May be obtained from the optician's or from J. Bell and Co., chemists, 338, Oxford Street, London.

This varnish flows easily from the brush, and sets quickly. For glycerin or other fluid mounts, the cover should be ringed with glycerin jelly before applying the varnish. This precaution is especially necessary with glycerin. This is the best varnish for fluid mounts known to me. It is soluble in ether or chloroform.

362. Black Japan—"Consists of asphalt, gum anime, amber, linseed oil, and oil of turpentine." ('Micro. Dict.')

363. Brunswick Black[1]—May be obtained from the optician's or oilshops. "Common Brunswick black is made by melting one pound of asphaltum, and then adding half a pound of linseed oil, and a quart of oil of turpentine.

"The best Brunswick black is prepared by boiling together a quarter of a pound of foreign asphaltum, and four and a quarter ounces of linseed oil which has been previously boiled with half an ounce of litharge until quite stringy; the mass is then mixed with half a pint of oil of turpentine, or as much as may be required to make it of a proper consistence. It is often improved by being thickened with lamp-black. . . If a little solution of india rubber in mineral naphtha be added to it, there is no danger of the cement cracking when dry." Carpenter states that without this addition it is brittle when dry. Brunswick black is soluble in oil of turpentine. A most useful cement, works easily and dries quickly. It can be recommended for turning cells.

364. Brunswick Black and Gold-Size (*Eulenstein's formula*[2]).

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[1] Beale, 'How to Work, &c.,' p. 49.

[2] Ibid.

—Equal parts of Brunswick black and gold-size with a very little Canada balsam.

365. Canada Balsam—Is sometimes used as a cement for fluid mounts, but cannot be recommended, as after a time it becomes so soaked with the fluid of the mounts (especially glycerin) as to lose its tenacity.

366. Dammar.—The same is the case with dammar. It is sometimes improved by the addition of a small quantity of solution of india rubber in naphtha.

367. Electrical Cement.[1]—Melt together 5 parts rosin, 1 part beeswax, and 1 part red ochre. The addition of 2 parts Canada balsam renders it much more strongly adhesive to glass.

368. Gold-Size[2]—Is found in commerce. It may be prepared by boiling 25 parts of linseed oil for three hours with 1 part of red lead and $\frac{1}{3}$ part of umber; then pour off. Successive portions of a finely-powdered mixture of equal parts of white lead and yellow ochre are then added to the oil, being well rubbed and mixed with it, until a tolerably thick liquid is formed; this must be once more thoroughly boiled.

369. Gutta-Percha Cement (*Harting's formula*[3]).—Gutta percha is to be cut into very small pieces and stirred at a gentle heat with 15 parts of oil of turpentine; the gritty insoluble matter which the gutta percha always contains is to be separated by straining through linen, and then 1 part of shellac is to be added to the solution, kept at a gentle heat and occasionally stirred. The mixture is to be kept hot until a drop when allowed to fall on a cool surface becomes tolerably hard.

The use of this cement is for attaching cells of gutta percha to the slide. When required for use, the mixture is to be heated, and a small quantity placed upon the slide; the slide itself is then to be heated.

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[1] 'Micro. Dict.,' "Cements."

[2] Ibid.

[3] Beale, 'How to Work, &c.,' p. 49.

370. Glue (Marine).—Found in commerce. Carpenter says the best is that known as G K 4.

It is prepared (Beale, p. 49) by dissolving, separately, equal parts of shellac and india rubber in mineral naphtha, and afterwards mixing the solutions thoroughly with the aid of heat. It may be rendered thinner by the addition of more naphtha.

It is soluble in ether, naphtha, or solution of potash. Its use is for attaching glass cells to slides, and for all cases in which it is desired to cement glass to glass.

371. India-Rubber and Lime French Cement.[1]—A quantity of india-rubber scraps is carefully melted over a slow fire in a covered iron pot. The mass must not be allowed to catch light. When it is quite fluid, lime, in a perfectly fine powder, having been slaked by exposure to the air, is to be added in small quantities at a time, the mixture being well stirred. When moderately thick, it is removed from the fire and well beaten in a mortar and moulded in the hands until of the consistence of putty. It may be coloured by the addition of vermilion or other colouring matter.

Beale says this cement is very valuable for mounting all large microscopical preparations. It never becomes perfectly hard, and therefore permits considerable alteration to take place in the volume of the liquid contained in the cell without the entrance of air. It also adheres very intimately to glass, even if it be perfectly smooth and unground. For mounting, the top of the cell is covered with a string of the cement formed by rolling between thumb and finger, and the cover pressed into it.

372. Knotting.—"Patent knotting" from oil and colour stores, exposed to the air until it has become of the proper consistency;—for mending cells and for preventing running-in of the finishing varnish (North, 'Microscopist,' ii (1882), p. 259).

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[1] Ibid., p. 53.

[2] 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 745.

373. Sealing-wax Varnish.[1]—Add enough spirit of wine to cover coarsely powdered sealing-wax, and digest at a gentle heat. This should only be used as a varnish, never as a cement, as it is apt to become brittle and to lose its hold upon glass after a time.

374. Shellac Varnish.[2]—Shellac should be broken into small pieces, placed in a bottle with spirit of wine, and frequently shaken until a thick solution is obtained. The 'Micro. Dictionary' says that the addition of 20 drops of castor-oil to the ounce is an improvement.

375. Venice Turpentine.[3]—Venice turpentine (Terebinthina veneta) is the liquid resinous exudation of *Abies larix.* It is seldom met with in a pure state. The following are the directions for preparing and using it given by C. B. Parker:[4]

Dissolve true Venice turpentine in enough alcohol, so that after solution it will pass readily through a filter, and, after filtering, place in an evaporating dish, and by means of a sand-bath, evaporate down to about three quarters of the quantity originally used. (The best way to tell when the evaporation has gone far enough is to drop some of the melted turpentine, after it is evaporated down to about three quarters its original volume, into cold water; if on being taken out of the water it is hard and breaks with a vitreous fracture on being struck with the point of a knife, cease evaporation and allow to cool.)

This cement is used for closing glycerin-mounts; it is applied in the following manner:—Square covers are used and superfluous glycerin is cleaned away from the edges in the usual way.

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[1] 'Micro. Dict.,' "Cements."

[2] Beale, 'How to Work, &c.,' p. 48.

[3] Garrod, 'Materia Medica,' p. 366.

[4] 'Amer. Mon. Mic. Journ.,' ii (1881), pp. 229-30. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 724.

The cement is then put on with a piece of wire bent at right angles (No. 10—12 wire is taken, and copper is the best, as it gives to the turpentine a greenish tinge); the short arm of the wire should be just the length of the side of the cover-glass. The wire is heated in a spirit-lamp, plunged into the cement, some of which adheres to it, and then brought down flat upon the slide at the margin of the cover. The turpentine distributes itself evenly along the side of the cover, and hardens immediately, so that the slide may be cleaned as soon as the four sides are finished. It is claimed for this cement that it is perfectly secure, very handy, and never runs in. Parker saw this cement, or a similar one known as *Venedischer Damarlack*, exclusively used for glycerin mounts in the Pathological Laboratory at Vienna.

376. Common Turpentine (*Csokor's formula*[1]).—Common resinous turpentine of commerce is put in small pieces to melt over a water-bath, then poured into a suitable vessel and allowed to cool. It should yield a brittle, dark brown mass, not yielding to the pressure of a finger. It is sometimes useful, in order to attain the right degree of hardness in the cold mass, to add a little resinous oil of turpentine to the melted mass, and then to evaporate for several hours over the water-bath.

The mass is applied to the edge of the cover by means of a heated bent wire, as described in the last paragraph. A coat of gold-size may be put on before the turpentine if desired. Like the last, it is used for glycerin mounts.

377. White Lead Cement (*Kitton's formula*[2]).—Equal parts of white lead, red lead, and litharge (all in powder), ground together with a little turpentine until thoroughly incorporated, then mixed with gold-size. The mixture should be thin enough to work with a brush. No more of the cement should be made than is required for present use, as it soon sets and becomes unworkable; but a stock of the materials may be kept ready-ground in a bottle.

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[1] 'Arch. Mik. Anat.,' xxi (1882), p. 353.

[2] 'Month. Mic. Journ.,' 1876, p. 221.

378. White Hard Varnish.[1]—Gum sandarac dissolved in spirit of wine, and mixed with turpentine varnish.

379. White Zinc Cement (*Stieda's formula*[2]).—Rub up oxide of zinc with turpentine, and add, stirring continually, for every drachm of the zinc oxide, 1 ounce of a solution of dammar in turpentine (of the consistency of thick syrup). This gives a *white* cement like Ziegler's. For a red cement, take, instead of zinc, *cinnabar*, and take 2 drachms of the metal for each ounce of the dammar solution. If the cement has become too thick with age, dilute with turpentine, ether, or chloroform.

380. Ziegler's White Cement.—Composition unknown. Is very much used on the Continent.

380 a. Gelatin Cement (*Marsh's formula*[3]).—Take half an ounce of Nelson's opaque gelatin, soak well in water, melt in the usual way, stir in 3 drops of kreasote, and put away in a small bottle. It is used warm.

When the ring of gelatin has become quite set and dry, which will not take long, it may be painted over with a solution of bichromate of potash made by dissolving 10 grains of the salt in an ounce of water. This should be done in the daytime, as the action of daylight is necessary to enable the bichromate to render the gelatin insoluble in water. The cover may then be finished with Bell's cement.

This process is particularly adapted for glycerin mounts.

[1] 'Micro. Dict.,' "Cements."

[2] 'Arch. Mik. Anat.,' ii (1866), p. 435.

[3] 'Section-cutting,' 2nd ed., p. 104.

CHAPTER XXVIII.

GELATINOUS AND ALBUMINOUS INJECTION-MASSES.

381. The art of injection does not seem to have made so much progress of late years as some of the sister branches of anatomical technic. Doubtless there has been advance in the working out of the details of the application of injection methods to certain special ends, but I am not aware that any invention has been made in this department that can claim to compare in importance with the recent improvements in the processes of preserving tissues by the employment of fixing agents, or in the processes of section-cutting and serial mounting. It is therefore unnecessary for me here to enter into any discussion of the principles on which the practice of injection is founded, but I may refer the reader who is unacquainted with these processes to the excellent exposition given in many technical works, and proceed at once to set out the *formulæ* for *injection-masses* recommended by the best workers, together with such account of the *processes* employed by them as may seem desirable.

It will probably be most convenient to set out the formulæ in an order roughly suggested by classifying the masses according to the nature of the vehicle for the colouring matter. This appears to be a more natural classification than that of Ranvier, who groups the masses according to their colouring material. I have therefore grouped my formulæ in the following order: Gelatinous-and albuminous-masses, fatty-masses, aqueous-masses, glycerin-masses, resinous-masses, collodion-masses.

382. Gelatin-Injection Vehicle (*Robin's formula*[1]).—Take some gelatin, of the sort known as "colle de Paris." (This gelatin is found in commerce in the form of thin sheets, marked with lozenge-shaped impressions of the cords which supported them whilst drying.) Soak it in cold water, then heat in water over a water-bath. One part of gelatin should be taken for every 7, 8, 9, or even 10 parts of water; it is a common error to employ solutions containing too much gelatin. The solution is now to be combined with the carmine colouring-mass given below, No. 383 ('Traité,' p. 33), or with the ferrocyanide of copper colouring-mass, No. 384 ('Traité,' p. 34), or with the Prussian blue colouring-mass, No. 385 ('Traité,' p. 35), or with the cadmium mass, No. 386 ('Traité,' p. 36), or with the Scheele's-green mass, No. 387 ('Traité,' p. 37); or with such anilin colours (*e.g.* blue, violet, yellow) as are insoluble in water, but will mix with the vehicle after having been dissolved in a little alcohol. (In the latter case alcohol must of course be avoided in the treatment of the injected organ, which may be preserved in glycerin.)

This mass, like all gelatin-masses, is liable to be attacked by mould if kept long; camphor and carbolic acid do not suffice to preserve them. The following plans for preserving them are recommended: The mass may be kept covered with a layer of alcohol in a closed vessel; the gelatin becomes opaque under the influence of the alcohol, but as soon as the alcohol is driven off by heat the gelatin regains its transparency. Or place the mass in a bottle and suspend above it, from the cork of the bottle, a sponge soaked with alcohol and a few drops of spirit of turpentine. The sponge must not touch the gelatin. This plan will preserve for months both the transparency and the colour of the mass.

(Chloral hydrate added to the mass will preserve it (Hoyer). (*See* below, No. 393.)

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[1] 'Traité,' p. 30.

The colouring-masses recommended for combination with the vehicle above described are made as follows:

383. Carmine Colouring-mass (*Robin's formula*[1]).—Rub up in a mortar 3 grammes of carmine with a little water and enough ammonia to dissolve the carmine. Add 50 grammes of glycerin, and filter.

Prepare 50 grammes of acid glycerin (containing 5 grammes of acetic acid for every 50 grammes of glycerin), and add it by degrees to the carmine glycerin, until a slightly acid reaction is obtained (as tested by very sensitive blue test-paper, moistened and held over the mixture).

One part of this mixture is to be added to 3 or 4 parts of the gelatin injection-vehicle (*ante*, Formula 382), or of the glycerin-gelatin, or glycerin-alcohol vehicle described below, Nos. 389 and 429.

384. Ferrocyanide of Copper Injection-mass (*Robin's formula*[2]):

Take—

(1)	Ferrocyanide of potassium (concentrated solution)	20 c.c.
	Glycerin	50 „
(2)	Sulphate of copper (concentrated solution)	35 „
	Glycerin	50 „

Mix (1) and (2) slowly, with agitation, at the moment of injecting combine with 3 volumes of vehicle (382, 389, or 429).

385. Blue Injection-mass (Prussian-Blue) (*Robin's modification of Beale's formula*[3]):

Take—

(A)	Sulphocyanide of potassium (sol. sat.) .	90 c.c.
	Glycerin	50 „
(B)	Liquid perchloride of iron at 30° .	3 „
	Glycerin	50 „

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[1] 'Traité,' p. 33.

[2] Ibid., p. 34.

[3] Ibid., p. 35.

Mix slowly and combine the mixture with 3 parts of vehicle (382, 389, or 429). It is well to add a few drops of HCl.

386. Cadmium Injection-mass (*Robin's formula*[1]):

Take—

Sulphate of cadmium (sol. sat.)	40 c.c.
Glycerin	50 ,,
and	
Sulphide of sodium (sol. sat.)	30 c.c.
Glycerin	50 ,,

Mix with agitation and combine with 3 vols. of vehicle (382, 389, or 429).

387. Scheele's Green Injection-mass (*Robin's formula*[2]):

Take—

Arseniate of potash (saturated solution)	80 c.c.
Glycerin	50 ,,
and	
Sulphate of copper (saturated solution)	40 c.c.
Glycerin	50 ,,

Mix and combine with 3 vols. of vehicle (382, 389, or 429.)

388. Anilin Injection-masses (*Robin*[3]).—The anilin colours have, for injections, the great fault of being soluble in alcohol; fuchsin is soluble in water, in alcohol, and in glycerin; it therefore cannot be employed with a gelatin or glycerin vehicle. Anilin blue, violet, yellow may be combined with these vehicles after dissolving in a small quantity of alcohol; and (alcohol being avoided for hardening purposes) the injected organs may be preserved in glycerin.

389. Glycerin-Gelatin Injection-masses (*Robin's formula*[4]).—Dissolve in a water-bath 50 grammes of French gelatin ("colle de Paris") in 300 grammes of water in which has been dissolved some arsenious acid; add of glycerin 150 grammes, and of carbolic acid a few drops.

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[1] Ibid., p. 36.

[2] Ibid., p. 37.

[3] Ibid., p. 37.

[4] Ibid., p. 32.

Combine this vehicle with one of Robin's colouring-masses (*ante*, 383, 384, 385, 386, 387).

This mass is not susceptible of change through long keeping.

390. Gelatin Injection-mass (Carmine) (*Ranvier's formula*[1]).—Take 5 grammes Paris gelatin, soak it in water for half an hour, or until quite swollen and soft; wash it; drain it; put it into a test-tube and melt it, in the water it has absorbed, over a water-bath. When melted add slowly, and with continual agitation, a solution of carmine in ammonia, prepared as follows:—2½ grammes of carmine are rubbed-up with a little water, and just enough ammonia, added drop by drop, to dissolve the carmine into a *transparent* solution.

When the carmine has been added to the gelatin you will have about 15 c.c. of ammoniacal solution of carmine in gelatin, if the operations have been properly performed. This solution is to be kept warm on the water-bath, whilst you proceed to neutralise it by adding cautiously, drop by drop, with continual agitation, a solution of 1 part of glacial acetic acid in 2 parts of water. (When the mass is near neutrality, dilute the acetic acid still further.) The instant of saturation is determined by the smell of the solution, which gradually changes from ammoniacal to sour. As soon as the sour smell is perceived, the addition of acetic acid must cease, and the liquid be examined under the microscope. If it contains a granular precipitate of carmine, too much acid has been added, and the mass must be thrown away.

Ranvier states that by practice the operator learns to attain to perfect neutralisation almost infallibly in this way; and that this is the only way to attain to it. Trust must not be put in certain formulæ that profess to indicate the proportions of ammonia and acetic acid necessary for neutralisation, on account of the variation in strength of the solutions of ammonia kept in laboratories. The method proposed by Frey of determining beforehand the quantity of a known acetic solution that is necessary for neutralisation of a given quantity of the ammonia employed, is not infallible because it often happens that commercial gelatin is acid; in which case, the proposed method would cause the operator to overpass the point of saturation.

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[1] 'Traité technique,' p. 116.

The mass having been perfectly neutralised is strained through new flannel.

391. Gelatin Injection-mass (Carmine) (*Gerlach's formula*[1]):

Carmine	5 parts
Water	4 „
Ammonia	0·50 „

Dissolve, and combine with following vehicle whilst warm:

Gelatin	6 parts
Water	8 „

Add to the mass a few drops of acetic acid.

Ranvier does not recommend this mass.

392. Gelatin Injection-mass (Carmine) (*Thiersch's formula*[2]):

Take of—

Carmine	1 part by weight
Liq. Am.	1 „
Water	3 „

Dissolve and filter.

Then take a solution of 1 part by weight of gelatin in 2 of water. Warm it to 25° R. in a water-bath, and mix 1 part of the carmine solution to 3 or 4 parts of the gelatin solution. Then add acetic acid, drop by drop, stirring continually, until no more ammonia remains free, as tested by the smell, by the non-formation of vapour when a glass rod moistened with acetic acid is held over the solution, and by moistened curcuma-paper held over it. (The carmine solution may be neutralised before mixing with the gelatin, if desired. The excess of ammonia may also be got rid of by evaporation at from 25° to 30° R.) Inject at a temperature of from 25°

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[1] Ranvier, 'Traité,' p. 118.

[2] 'Arch. Mik. Anat.,' i (1865), p. 148.

to 35° R., put the preparation to cool on ice as soon as the operation of injecting is completed, harden in alcohol. Very delicate objects should be put at once into alcohol previously cooled by means of ice.

393. Gelatin Injection-mass (Carmine) (*Hoyer's formula*[1]). —Take a concentrated gelatin solution and add to it a corresponding quantity of the neutral carmine (staining solution) (Formula No. 58). Digest in a water-bath until the dark violet-red colour begins to pass into a bright-red tint. Then add 5—10 per cent. by volumes of glycerin and at least 2 per cent. by weight of chloral in a concentrated solution. After passing through flannel, it can be kept in an open vessel under a bell-glass.

394 Gelatin Injection-mass (Carmine) (*Carter's formula*[2]): Take—

Carmine	1 drachm.
Liq. ammon. fort. . . .	2 drachms.
Glacial acetic acid . . .	1 drachm 26 minims.
Solution of gelatin (1 to 6 of water)	2 ounces.
Water	1½ ounces.

Dissolve the carmine in the ammonia and water; filter if necessary. Add one ounce and a half of the gelatin solution hot. Mix the acetic acid with the remaining half ounce of gelatin, and drop this mixture, little by little, into the carmine and gelatin solution, stirring briskly all the while.

395 Gelatin Injection-mass (Prussian Blue) (*Thiersch's formula*[3]):

Take—

(1) A solution of 1 part gelatin in 2 parts water.

(2) A saturated aqueous solution of sulphate of iron.

(3) A saturated aqueous solution of red prussiate of potash.

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[1] 'Biol. Centralbl.,' ii (1882), pp. 19-22. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 142.

[2] Beale, 'How to Work, &c.,' 1865, p. 113.

[3] 'Arch. Mik. Anat.,' i (1865), p. 148.

(4) A saturated aqueous solution of oxalic acid.

Now (A) mix 12 c.c. of the iron solution with one ounce of the gelatin solution at the temperature of 25° R.

Then (B) mix, at the same temperature, 24 c.c. of the prussiate solution with two ounces of the gelatin solution.

(C.) To the latter mixture add first 24 c.c. of the oxalic acid solution, stir well, and then add the gelatin and iron mixture (A). Stir continually, keeping the temperature at from 20° to 25° R. until the whole of the Prussian-blue is precipitated. Finally, heat over a water-bath to about 70° R. and filter through flannel.

396. Gelatin Injection-mass (Berlin-Blue) (*Brücke's formulæ*[1]).—Brücke uses exclusively that form of Berlin-blue to which Berzelius assigned the formula $[K_2+(FeCy_2)Cy_2] + [Fe_3+(2Fe_2Cy_3)Cy_3]$.

He first prepared it by taking a 10 per cent. solution of ferrocyanide of potassium, and precipitating by means of a dilute solution of sesquichloride of iron (taken in such a quantity as to contain just half as much chlorine as is necessary for the decomposition), and washing the precipitate on the filter until solubility is attained.

Later on, he employed a greater excess of ferrocyanide, and took just so much dilute solution of chloride of iron that the weight of the dry chloride employed came to $\frac{1}{10}$th or $\frac{1}{8}$th of that of the ferrocyanide. The precipitate was washed on a filter (using the filtrate to wash with), until nothing but a clear yellow liquid filtered off, then washed with water, until the water began to run off blue, then dried, pressed between blotting-paper in a press, the resulting mass broken in pieces, and dried by exposure to the air.

A cheaper method is the following:

Make a solution of ferrocyanide of potassium containing 217 grammes of the salt to 1 litre of water.

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[1] Ibid., p. 87.

Make a solution of 1 part commercial chloride of iron in 10 parts water.

Take equal volumes of each, and add to each of them twice its volume of a cold saturated solution of sulphate of soda. Pour the chloride solution into the ferrocyanide solution, stirring continually. Wash the precipitate on a filter until soluble, and treat as above described.

The concentrated solution of the colouring matter is to be gelatinised with just so much gelatin that the mass forms a jelly when cold. It is melted by means of a temperature of about 60° C. and injected with a warm syringe, but there is no need to warm the subject. The injected pieces are hardened in alcohol of at least 94 per cent.; sections are cleared with *resinous* turpentine (prepared by exposing turpentine to the air in large vessels), and mounted in cells in dammar varnish. They may be stained with carmine if desired. If it be wished to avoid treatment with alcohol, chromic acid and bichromate of potash and sulphate of soda may be employed; but glycerin should be avoided for mounting as it causes the colour to become pale.

The object of employing resinous turpentine is to prevent the brittleness caused in sections by the use of ordinary oil of turpentine.

397. Gelatin Injection-mass (Berlin-Blue) (*Müller's formula*[1]).—Dissolve 1 part gelatin in 8 parts of a "not too concentrated" solution of so-called soluble Berlin-blue.

(The Berlin-blue is obtained by precipitation from a solution of ferrocyanide of potassium (which must be in excess), and washing the precipitate until it becomes soluble.)

398. Gelatin Injection-mass (Berlin-Blue) (*Hoyer's formula*[2]).—The filtered and not too much washed precipitate of soluble Berlin-blue is brought in a little water on to a Graham's dialyser, and the external water changed until the solution begins to pass through the parchment. Dilute the solution and filter through filter-paper, an operation which becomes easy *after* dialysis. The solution may be injected pure (for lymphatics, for instance) or may be combined with gelatin. To do this, warm the solution almost to boiling-point, and add gradually a warm thin solution of gelatin until coagulation begins to set in. Strain through wetted flannel.

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[1] Ibid., p. 150.

[2] Ibid., xiii (1876), p. 649.

399. Gelatin Injection-mass (Prussian-Blue) (*Ranvier's formula*[1]).—Twenty-five parts of a concentrated aqueous solution of soluble Prussian-blue (prepared as directed below), mixed with 1 part of solid gelatin.

The mixture of the Prussian-blue with the vehicle is effected in the following manner:

Weigh the gelatin, soak it in water for half an hour or an hour, wash it, and melt it in a test-tube, in the water it has absorbed, by heating over a water-bath. Put the solution of Prussian-blue into another test-tube, and heat it on the same water-bath as the gelatin, so as to have the two at the same temperature. Pour the gelatin gradually into the Prussian-blue solution, stirring continually with a glass rod. Continue stirring until the disappearance of the curdy precipitate that forms at first. (Some gelatins produce a *persistent* precipitate; these must be rejected; but it must be borne in mind that the precipitate that invariably forms in even the best gelatins disappears if the heating be continued. It is essential to remember this when preparing Prussian-blue and gelatin mass.) As soon as the glass rod has ceased to show blue granulations on its surface on being withdrawn from the liquid, it may be concluded that the Prussian-blue is completely dissolved. Filter through new flannel, and keep the filtrate at 40° over a water-bath until injected.

The soluble Prussian-blue for the above mass is prepared as follows:

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[1] 'Traité,' pp. 119-121.

400. Soluble Prussian-Blue for Injection-masses (*Ranvier's formula*[1]).—Make a concentrated solution of sulphate of peroxide of iron in distilled water, and pour it gradually into a concentrated solution of yellow prussiate of potash. There is produced a precipitate of insoluble Prussian-blue. (An excess of prussiate of potash ought to remain in the liquid; in order to ascertain whether this is the case take a small quantity of the liquid and observe whether a drop of sulphate of iron still precipitates it.) Filter the liquid through a felt strainer, underneath which is arranged a paper filter in a glass funnel. The liquid at first runs clear and yellowish into the lower funnel; distilled water is then poured little by little on to the strainer; gradually the liquid issuing from the strainer acquires a blue tinge, which, however, is not visible in that which issues from the lower filter. Distilled water is continually added to the strainer for some days until the liquid begins to run off blue from the second filter. The Prussian-blue has now become soluble. The strainer is turned inside-out and agitated in distilled water; the Prussian-blue will dissolve if the quantity of water be sufficient.

The solution may now be injected just as it is, or it may be kept in bottles till wanted, or the solution may be evaporated in a stove, and the solid residuum put away in bottles.

For injections, if a simple aqueous solution be taken, it should be *saturated.* Such a mass never transudes through the walls of vessels. Or, it may be combined with one fourth of glycerin, or with the gelatin vehicle above described.

401. Gelatin Injection-mass (Berlin-Blue) (*Hoyer's formula*[2]).—Mix a small quantity of a *very dilute* and warm solution of Berlin-blue with an equally small quantity of a moderately dilute gelatin solution. By this means a clear homogeneous blue solution is obtained. This is mixed with larger quantities of concentrated warm gelatin-solution, with the gradual addition of now only a moderately dilute solution of Berlin-blue. A homogeneous transparent saturated mass is thus produced. The addition of chloral and glycerin (*see ante*, Hoyer's formula for carmine gelatin mass, No. 393) enables it to be kept for a long time.

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[1] Ibid., p. 119.

[2] 'Biol. Centralbl.,' ii (1882), pp. 19-22. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 142.

402. Gelatin Injection-mass (Lead Chromate) (*Thiersch's formula*[1]):

Make—

(A) A solution of 1 part gelatin in 2 parts water.

(B) A solution of 1 part neutral chromate of potash in 11 parts water.

(C) A solution of 1 part nitrate of lead in 11 parts water.

Mix 4 parts of the gelatin solution with 2 parts of the lead solution, and in another vessel mix 4 parts gelatin solution with 1 part of the chromate solution. Heat both the mixtures to 25° R., mix them together with continual stirring until all the chromate of lead is precipitated; heat over a water-bath to 70° R. and filter through flannel.

403. Gelatin Injection-mass (Transparent Green) *Thiersch's formula*[2]).—The blue injection-mass (formula No. 395) mixed in various proportions with the yellow mass (No. 402) gives transparent green masses of different hues.

404. Chrome-Yellow Injection-mass (*Hoyer's formula*[3]):

Take—

- One volume of a solution of gelatin containing 1 part of gelatin to 4 of water.
- One volume of cold saturated solution of bichromate of potash.
- And one volume cold saturated solution of sugar of lead (neutral plumbic acetate).

Filter the gelatin solution through flannel, and mix in the bichromate solution. Then *warm almost to boiling point*, and add gradually the (warmed) sugar of lead solution. Allow the mass to cool down to body temperature and inject at once. Another mode of preparation is as follows: Mix the sugar of lead solution with part of the gelatin solution, mix the bichromate solution with the remaining gelatin solution, heat the latter mixture, and pour into it the former mixture (gradually), stirring continually.

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[1] 'Arch. Mik. Anat.,' i (1865), p. 149.

[2] Ibid.

[3] Ibid., iii (1867), p. 136.

If the solutions are mixed at a low temperature a lumpy granular precipitate is formed. Further, when solution of sugar of lead is added to a *hot* solution of bichromate of potash, a rich orange red precipitate is obtained; whilst if the solutions be mixed *cold*, the precipitate is bright yellow.

If the solutions of the two salts be kept ready prepared, the injection-mass may be mixed in less than a quarter of an hour. Its advantages are, that on account of the extremely fine state of division of the precipitate the mass is almost transparent, and runs so freely that even lymphatics may be perfectly injected with it, whilst its intensity of colour makes the vessels much more distinct than the very pale mass of Thiersch (No. 402). It is also easier to manage than Thiersch's mass, as it does not solidify so quickly. It shows well in the vessels by reflected, as well as by transmitted, light.

405. Gelatin Injection-mass (Nitrate of Silver) (*Ranvier's formula*[1]).—Concentrated solution of gelatin, 2, 3, or 4 parts; 1 per cent. nitrate of silver solution, 1 part.

406. Gelatin Injection-mass (Yellow.—Silver Nitrate) (*Hoyer's formula*[2]).—"A concentrated solution of gelatin is mixed with an equal volume of a 4 per cent. solution of nitrate of silver and warmed. To this is added a very small quantity of an aqueous solution of pyrogallic acid which reduces the silver in a few seconds; chloral and glycerin are added as before" (*see ante*, Hoyer's formula for carmine-gelatin, No. 393).

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[1] 'Traité,' p. 123.

[2] 'Biol. Centralbl.,' ii (1882), pp. 19, 22. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 142.

This mass is yellow in the capillaries and brown in the larger vessels. It does not change either in alcohol, chromic or acetic acid, or bichromate of potash, &c.

407. Gelatin Injection-mass (Green) (*Hoyer's formula*[1]).—"Blue and yellow masses mixed give a very useful green."

408. Gelatin Injection-mass (White) (Carbonate of Lead) (*Hartig's formula*[2]).—Dissolve 125 grammes of acetate of lead in so much water that the whole shall weigh 500 grammes.

Dissolve 95 grammes of carbonate of soda in so much water that the whole shall weigh 500 grammes.

Take equal volumes of the two solutions, and add two volumes of gelatin-solution.

409. Gelatin Injection-mass (White) (Sulphate of Baryta) (*Frey's formula*[3]).—Put into a tall glass cylinder 125 to 185 grammes of cold saturated solution of chlorate of baryta. Add drop by drop, very carefully, sulphuric acid. Allow the precipitate that forms to settle for twelve hours, then decant almost all the clear supernatant liquid. The remaining mucilaginous mass containing the precipitate is to be mixed with an equal part of concentrated gelatin-solution.

Frey states that this is a very finely-grained mass. Injected organs may be preserved in chromic acid.

410. Gelatin Injection-mass (White) (Chloride of Silver) (*Teichmann's formula*[4]).—"Take 3 parts of nitrate of silver dissolved in the gelatin solution, and add 1 part of common salt."

The mass is very fine-grained, and is not decomposed by chromic acid; the disadvantage of it is that it blackens under the influence of light and of sulphurous solutions.

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[1] L. c.

[2] Frey, 'Le Microscope,' p. 190.

[3] Ibid.

[4] Ibid., p. 191.

411. Gelatin Injection-mass (Carmine) (*Davies and Dale's formula*[1]):

Take—

180 grains best carmine.
½ fluid ounce of ammonia, commercial strength, viz. 0·92 or 15° ammonia meter.
3 or 4 ounces water.

Digest without heat until the carmine is dissolved. Filter and add water until the whole is equal to sixteen ounces.

Dissolve 600 grains potash alum in ten fluid ounces of water, and add, under constant boiling, solution of carbonate of soda until a slight permanent precipitate is produced. Filter and add water up to sixteen ounces. Boil and add the solution to the carmine solution and shake vigorously for a few minutes. A drop of the liquid placed upon white filtering paper should show no coloured ring. If much colour is in solution the whole must be rejected. If the precipitation is complete or nearly so, shake vigorously for at least half an hour, and allow the fluid to stand until quite cold. The shaking must then be renewed for some time, and the bottle (a Winchester quart bottle) filled up with water.

Allow the precipitate to settle for a day, and draw off the clear supernatant fluid with a syphon. Repeat the washing until the clear liquid gives little or no precipitate with chloride of barium. So much water must be left with the colour at last that it shall measure forty fluid ounces.

For the injecting fluid take twenty-four ounces of the coloured liquid and three ounces of gelatin. Allow them to remain together twelve hours and dissolve over a water-bath. Strain through fine muslin.

This formula is the outcome of experiments made to find a transparent mass free from all tendency to extravasation. The authors consider they have succeeded.

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[1] Davies, 'On Preparing and Mounting Microscopic Objects,' p. 138.

412. Gelatin Injection-mass (Carmine) (*Fol's formula*[1]).—The three following methods of preparation have the advantage of producing masses that can be kept in the *dry state* for an indefinite length of time. (Fol finds that the addition of chloral hydrate to wet masses is not an efficient preservative.)

One kilog. of Simeon's photographic gelatin[2] is soaked for a couple of hours, until thoroughly soft, in a small quantity of water. The water is then poured off and the gelatin melted over a water-bath, and one litre of concentrated solution of carmine in ammonia is poured in with continual stirring. (The carmine solution is prepared by diluting strong solution of ammonia with three or four parts of water and adding carmine to saturation; the undissolved excess of carmine is removed by filtration just before the solution is added to the gelatin.)

To the mixture of gelatin and carmine, which should have a strong smell of ammonia, sufficient acetic acid is now added to turn the dark-purple colour of the mixture into the well-known blood-red hue. Exact neutralisation is not necessary. The mass is set aside until it has become firm, and is then cut up into pieces which are tied up in a piece of tulle or fine netting. By means of energetic compression with the hand under water (it must be *acidulated* water, 0·1 per cent. acetic acid, otherwise the carmine will wash out, cf. 'Journ. Roy. Mic. Soc.,' iv, part 3 (1884), p. 474) the mass is driven out through the meshes of the stuff in the shape of fine strings, which are washed for several hours in a sieve placed in running water in order to free them from any excess of acid or ammonia.

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[1] 'Zeit. wiss. Zool.,' xxxviii (1883), p. 492.

[2] This gelatin may be obtained either from the ordinary furnishers of articles used in photography, or direct from Simeon's Gelatin-fabrik Winterthur, Switzerland. Two sorts, a hard and a soft, are sold; the softer is to be preferred on account of its lower point of fusion. Probably the photographic gelatins of Hinrichs, of Frankfurt, and of Coignet, of Paris, would answer equally well.

The strings are then again melted, and the molten mass is poured on to large sheets of parchment paper soaked with paraffin, and the sheets are hung up to dry in an airy place. When dry the gelatin can easily be separated from the sheets, and may be cut into long strips with scissors and put away, protected from dust and damp, until wanted for use. In order to get the mass ready for use, all that is necessary is to soak the strips for a few minutes in water and melt them over a water-bath.

413. Gelatin Injection-mass (Blue) (*Fol's formula*[1]).—A modification of Thiersch's formula, No. 395. 120 c.c. of a cold saturated solution of sulphate of iron are mixed with 300 c.c. of the warm gelatin solution. In a separate vessel 600 c.c. of the gelatin solution are mixed with 240 c.c. of a saturated solution of oxalic acid, and 240 c.c. of a cold saturated solution of red prussiate of potash are added to the mixture. The first mixture is now gradually poured into the second, with vigorous shaking, the whole is warmed for a quarter of an hour over a boiling water-bath, the mass is allowed to set, is pressed out into strings through tulle or netting as described for the carmine mass, *supra*, and the strings are washed and spread out to dry on the prepared paper. (It is necessary to dry the strings without remelting in this case, because the mass does not readily melt without the addition of oxalic acid.) In order to prepare the mass for injection, the strings are put to swell up in cold water, and then warmed with the addition of enough oxalic acid to allow of complete solution.

414. Gelatin Injection-mass (Black) (*Fol's formula*[2]).—Five hundred grammes of gelatin are soaked, and allowed to swell up, in two litres of water in which 140 grammes of common salt have previously been dissolved; the mass is melted over a water-bath, and a solution of 300 grammes of nitrate of silver in a litre of water is gradually added, with vigorous shaking. (If it be desired to have an extremely fine-grained mass, both the solutions should be diluted with three or four volumes of water.) The mass is pressed out into strings as before, and the strings are stirred up, in clear daylight, with the following mixture: 1½ litres of cold saturated solution of potassic oxalate to 500 c.c. of cold saturated solution of sulphate of iron. As soon as the whole mass is thoroughly black the operation is at an end. The strings are then washed for several hours, remelted, and poured on to the prepared paper.

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[1] 'Zeit. wiss. Zool.,' xxxviii (1883), p. 494.

[2] Ibid.

This injection appears dark sepia-brown by transmitted light. If a grey-black hue be preferred, 240 grammes of bromide of potassium should be substituted for the sodium chloride in the first solution.

Fol warns against the employment of anilin colours for these injection-masses. They diffuse out during injection, or, if not, during the subsequent treatment of the preparations. Chrome colours are not applicable to dry masses, as treatment with chromic compounds causes the gelatin sooner or later to become insoluble.

415. Glue Cold Injection-mass (Logwood) (*Joseph's formula*[1]).—"Glue liquid when cold, coloured with the violet extract of logwood reduced with alum." This mass has similar properties to the white-of-egg mass, No. 416.

416. White-of-Egg Injection-mass (Carmine) (*Joseph's formula*[2]).—"Filtered white of egg, diluted with 1 to 5 per cent. of carmine solution. . . . This mass remains liquid when cold; it coagulates when immersed in dilute nitric acid, chromic or osmic acids, remains transparent, and is sufficiently indifferent to reagents."

For Invertebrates.

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[1] 'Ber. naturw. sect. Schles. Ges.,' 1879, pp. 36-40. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 274.

[2] Ibid.

CHAPTER XXIX.

FATTY, AQUEOUS, AND GLYCERIN INJECTION-MASSES.

FATTY MASSES.

417. Linseed-oil Cold Injection-mass (Vermilion) (*Teichmann's formula*[1]) :

Prepared chalk . . .	5 gr.
Vermilion	1 „
Linseed oil	0·9 to 1 c.c.
Carbon disulphide . . .	0·75 c.c.

Mix. The linseed oil is first boiled for eight to ten hours; no lead compounds are added to it. "The injection must be made with a syringe, such as those for injecting gutta percha, in which the piston is impelled by a screw arrangement, ordinary hand pressure not being powerful enough.

"With this mass, which is fluid when cold, the finest ramifications of vessels may be readily and with certainty filled. The mass soon stiffens, owing partly to transudation and partly to evaporation of the carbon disulphide, so that it does not ooze from vessels which may be cut through; it remains soft for a time, and is as hard as stone when the preparation is finished."

The following are differently coloured variations of this mass, all of them consisting "essentially of finely-powdered materials and linseed oil made up to the consistency of putty, and altered to that of honey or syrup, as required, by volatile liquids, such as ether and carbon disulphide." For the injection of entire subjects by the aorta, Teichmann uses first of all a thinner mass, consisting of chalk 500 gr., vermilion 100 gr., linseed oil 120 c.c., carbon disulphide 150 c.c.; he then employs a stiffer preparation of chalk 1000 gr., vermilion 200 gr., linseed oil 200 c.c., carbon disulphide 100 c.c.

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[1] 'S. B. Math. Kl. Krakau. Akad.,' vii, pp. 108-158. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), pp. 125 and 716.

418. **Linseed-oil Cold Injection-mass (White)** (*Teichmann's formula*[1]).—Zinc white 20 gr., linseed oil 3 c.c., ether 2 cc. For injection of lymphatics.

(By addition of colouring matters this mass forms other combinations.)

419. **Linseed-oil Cold Injection-mass (Blue)** (*Teichmann's formula*[2]).—Zinc white 15 gr., ultramarine 1 gr., linseed oil 2 to 2½ c.c., carbon disulphide 1 c.c.

420. **Axunge and Spermaceti Injection-mass** (*Robin's formula*[3]):

Axunge	40 grammes.
Spermaceti . . .	40 ,,
White wax . . .	10 ,,
Spirit of turpentine .	15 ,,

Melt at a gentle heat, and add an oil-ground pigment.

421. **Suet Injection-mass** (*Robin's formula*[4]).—Take suet and add to it "a certain quantity" of spirit of turpentine, or of axunge. Combine with a pigment as before.

The pigments recommended by Robin are vermilion (best mixed with a little white), Prussian-blue mixed with 5 parts of white, chrome-yellow, or "blanc d'argent."

422. **Wax and Oil Cold Injection-mass** (*Griesbach's formula*[5]). —Made "by heating equal parts of white and yellow wax, and dissolving in oil of turpentine; after cooling the solution is mixed with olive or rapeseed oil in which sulphate of lead

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[1] L. c.

[2] L. c.

[3] 'Traité,' p. 23.

[4] Ibid.

[5] 'Arch. Mik. Anat.,' xxi (1882), pp. 824-7. 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 297.

has been ground up. . . . Sulphate of barium or iodide of lead may be substituted for the sulphate of lead. By the addition of spermaceti to the solution in oil of turpentine the mass can be made thinner."

AQUEOUS INJECTION-MASSES.

423. Red Injection-fluid (*Brücke's formula*[1]).—A concentrated solution of ferrocyanide of potassium is injected into the arteries until it flows away from the veins almost unmixed with blood. As much of it as possible is allowed to drain away from the veins and from the nozzle of the syringe, which is left open. A concentrated solution of sulphate of copper (which must be free from iron) is then thrown in. After twenty-four hours the specimens may be prepared as desired. The colour obtained by this method being red, carmine-staining is not very suitable. An advantage of the method is that (in cool weather at all events) it is not necessary to have recourse to preservative liquids during the preparation of the specimens, as the liquids injected are themselves powerful preservatives.

424. Ammoniacal Silver-Nitrate Injection-fluid (*Hoyer's formula*[2]).—To a solution of nitrate of silver of known concentration add ammonia until the precipitate is seen to be just barely re-dissolvable; then dilute the solution until it contains from 0·75 to 0·5 per cent. of the nitrate of silver. Inject this solution. (A concentrated solution of gelatin may be injected afterwards if desired.)

The advantage that this ammoniacal solution of silver has over the pure nitrate is that it stains the endothelium alone, leaving other tissues unstained; if used in the ordinary way for impregnation of membranes it will be found to leave connective tissue colourless.

425. Aqueous Berlin-Blue Cold Injection-fluid (*Müller's for-*

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[1] 'Arch. Mik. Anat.,' ii (1866), p. 91.

[2] Ibid. xiii (1876), p. 649.

mula[1]).—Precipitate a concentrated solution of Berlin-blue (No. 397) by means of 90 per cent. alcohol.

The precipitate is very finely divided; the fluid is *perfectly neutral* and much easier to prepare than the formula of Beale or Richardson.

426. Aqueous Injection-fluid (Prussian-Blue) (*Ranvier's formula*[2]).—A saturated aqueous solution of soluble Prussian-blue, prepared as directed for the gelatin injection-mass, formula No. 400.

427. Aqueous Injection-fluid (Carmine) (*Emery's formula*[3]).—To a 10 per cent. ammoniacal solution of carmine is added acetic acid, with continual stirring, until the colour of the solution changes to blood-red through incipient precipitation of the carmine. The supernatant clear solution is poured off, and injected cold without further preparation. The injected organs are thrown at once into strong alcohol to fix the carmine. For injection of Fishes.

428. Glycerin Injection-fluid (Prussian-Blue) (*Ranvier's formula*[4])—Consists of the aqueous Prussian-blue injection-fluid, *ante* No. 426, mixed with one fourth of glycerin.

429. Glycerin Injection-fluid (Carmine) (*Robin's formula*[5])—Consists of the following vehicle :

Glycerin	2 parts.
Alcohol	1 „
Water	1 „

Combined with one third or one fourth its volume of the carmine colouring-mass, *ante* formula No. 383.

430. Glycerin Injection-fluid (Mahogany) (*Robin's formula*[6])—Consists of the precedent vehicle combined, at the moment of injecting, with one third its volume of the mahogany colouring-mass, formula No. 384.

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[1] Ibid., i (1865), p. 150.

[2] 'Traité,' p. 120.

[3] 'Mitth. Zool. Stat. Neapel,' ii (1881), p. 21.

[4] 'Traité,' p. 120.

[5] Ibid., p. 33.

[6] Ibid., p. 34.

431. Glycerin Injection-fluid (Prussian-Blue) (*Robin's formula*[1])—Consists of the precedent vehicle combined with one third its volume of the Prussian-blue colouring-mass, formula No. 385.

432. Glycerin Injection-fluid (Cadmium) (*Robin's formula*[2])—Consists of the precedent vehicle combined with one third its volume of the cadmium colouring-mass, formula No. 386.

433. Glycerin Injection-fluid (Scheele's-Green) (*Robin's formula*[3])—Consists of the precedent vehicle combined with one third its volume of the Scheele's-green colouring-mass, formula No. 387.

434. Glycerin Injection-fluid (*Wywodsen's formula*[4]).—"D. Wywodsen has obtained admirable results by using thymol. The proportions are: thymol 5 parts, alcohol 45, glycerin 2160, water 1080."

435. Glycerin and Wood-Naphtha Injection-mass (Prussian-Blue) (*Beale's formula*[5]):

Take—

Glycerin	2 ounces.
Wood-naphtha or pyro-acetic spirit .	1½ drachms.
Spirit of wine	1 ounce.
Ferrocyanide of potassium . .	12 grains.
Tincture of sesquichloride of iron (the muriated tincture of iron, B. P.) .	1 drachm.
Water	3 ounces.

Dissolve the ferrocyanide of potassium in 1 ounce of the glycerin, mix the tincture of iron with the other ounce. Add the iron mixture drop by drop to the ferrocyanide solution, shaking all the time. (When thoroughly mixed, no flocculi should be perceptible.) Then mix the naphtha with the spirit, add the water very gradually, and shake the whole.

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[1] 'Traité,' p. 35.

[2] Ibid., p. 36.

[3] Ibid., p. 37.

[4] 'St. Petersb. Med. Wochenschrift,' No. 51. 'Journ. Roy. Mic. Soc.' (N.S.), ii (1882), p. 717.

[5] 'How to Work, &c.,' 3rd ed. (1865), p. 113.

For very fine injections, half the quantity of iron and ferrocyanide may be taken.

436. Acidulated Glycerin (HCl) Injection-mass (Prussian-Blue) (*Beale's formula*[1]) :

Price's glycerin	2 fluid ounces.
Tinct. of sesquichloride of iron .	10 drops.
Ferrocyanide of potassium .	3 grains.
Strong hydrochloric acid . .	3 drops.
Water	1 ounce.

Proceed as directed above, dissolving the ferrocyanide in one half of the glycerin, the iron in the other, and adding the latter drop by drop to the former. Finally add the water and HCl. Two drachms of alcohol may be added to the whole if desired.

I consider this a most admirable formula. I possess some of this mass prepared more than three years ago, in which not the smallest flocculus has made its appearance. The Prussian-blue appears to be in a state of true solution. The mass runs well, and has not so much tendency to exude from cut capillaries as might be supposed.

436 a. Neutral Glycerin Prussian-Blue (*Beale's formula*[2]) :

Common glycerin	1 ounce.
Spirits of wine	1 ,,
Ferrocyanide of potassium . . .	12 grains.
Tincture of perchloride of iron . .	1 drachm.
Water	4 ounces.

Proceed as directed above, dissolving the ferrocyanide in 1 ounce of the water and glycerin, and adding the tincture of iron to another ounce. "These solutions should be mixed together *very gradually* and well shaken in a bottle, *the iron being added to the solution of the ferrocyanide of potassium.*

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[1] 'How to Work, &c.,' 3rd edit. (1865), p. 200; 4th ed., p. 296.

[2] Ibid., 4th ed., p. 93.

Next, the spirit and the water are to be added very gradually, the mixture being constantly shaken."

"*The water*" spoken of in the last sentence appears to mean the remaining 3 ounces of water that were not mixed with the glycerin at first.

Injected specimens should be preserved in acidulated glycerin, otherwise the colour may fade.

436b. Neutral Glycerin (Turnbull's-Blue) (*Richardson's formula*[1]).—Ten grains of pure sulphate of iron are to be dissolved in an ounce of glycerin, and 32 grains of ferridcyanide of potassium in a small proportion of water and then mixed with glycerin. Mix these two solutions gradually and add the other ingredients as directed for the Prussian-blue (last formula). Beale finds these proportions too large, and prefers the following:

Ferridcyanide of potassium . .	10 grains.
Sulphate of iron	5 ,,
Water	1 ounce.
Glycerin (Price's)	2 ,,
Alcohol	1 drachm.

It is believed by some that Turnbull's-blue is less liable to fade than the Prussian-blue. The colour is brighter.

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[1] Beale, 'How to Work, &c.,' 4th ed., p. 94.

CHAPTER XXX.

RESINOUS AND COLLODION INJECTION-MASSES.

437. Shellac Injection-mass (*Hoyer's formula*[1]).—Place a quantity of good shellac in a wide-necked flask and add just enough alcohol (of about 80 per cent. strength) to cover the shellac. Leave it for twenty-four hours, and then warm it in a water-bath to complete the solution. When cool, dilute, if necessary, with alcohol to the consistency of a thin syrup and strain through moderately thick muslin. The solution thus obtained may be coloured by the addition of anilin colours in (filtered) concentrated alcoholic solution, or of granular pigments suspended in alcohol. Of these cinnabar gives the finest colouration, and may be employed for corrosion preparations (anilin colours may also be used for this purpose, but then they are not permanent). Berlin-blue and yellow sulphide of arsenic are useful. A mixture of the two gives green. Freshly-precipitated sulphide of cadmium gives a fine permanent yellow. The pigments should be rubbed up to fine powder with water, and alcohol added; let the mixture settle, pour off the dilute alcohol and add strong alcohol. Shake in a flask, by which means the coarser particles are brought to the bottom of the liquid, and at this moment pour off the supernatant fluid which contains the finer particles only. Add this to the shellac solution and strain through muslin. For very minute injections dilute the mixture with alcohol, filter through filter-paper on a covered funnel, and evaporate down to the desired consistency. Common moist water colours, such as are sold in tin tubes, may be employed; they are to be well washed through several changes of water to get rid of the medium with which the pigments are mixed, and then suspended in alcohol as above directed. (These are to be recommended for injections into the blood of living animals.)

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[1] 'Arch. Mik. Anat.,' iii (1876), p. 645.

The shellac-solution is not attacked by hydrochloric acid; hence its applicability to corrosion preparations. To correct the brittleness of the corroded mass it is well to add to the injection-fluid some 5 per cent. of a filtered alcoholic solution of Venetian turpentine. This may also be of use for preparations that are not to be corroded. For corrosion, concentrated (fuming) hydrochloric acid may be used, and small objects left in it for one day, large ones many days or even weeks.

For hardening injections, of which it is desired to cut sections, chromic acid may be used, or a mixture of chromic and hydrochloric acid (1 part of each to 250—500 parts water). Sections are best mounted in glycerin.

438. Turpentine Injection-mass (*Robin's formula*[1]).—"Fine oil-ground oil-colours, diluted with turpentine."

The colours recommended are vermilion, Prussian-blue, chrome yellow, flake white. The Prussian-blue should be mixed with white, in the proportion of one part of the blue to five of the white. Vermilion too is better mixed with a little white.

439. Sealing-wax Injection-mass (*Robin's formula*[2]).—The sealing-wax called "cire d'Espagne," dissolved to saturation in alcohol.

440. Asphaltum Injection-mass (juice-canals of cartilage) (*Budge's formula*[3]).—A large quantity of asphaltum has benzol poured on it, and is allowed to stand for several days, and then preserved for use. Before injecting add $\frac{1}{3}$ to $\frac{1}{2}$ benzol, and filter. Chloroform and turpentine may also be used as solvents. Used for injecting the juice-canals of cartilage by the method described l. c., or by puncture.

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[1] 'Traité,' p. 23.

[2] Ibid.

[3] 'Arch. Mik. Anat.,' xiv (1877), p. 70.

441. Celloidin Injection-mass (*Schiefferdecker's formulæ*[1]).—1. **Asphalt-celloidin** is the best of these injections. To prepare it—

Pulverise asphalt in a mortar, and put it for twenty-four hours into a well-closed vessel with some ether, shaking occasionally. After the twenty-four hours pour off the ether into another vessel, and dissolve in it small pieces of celloidin until the solution is of the consistency of one of the thicker fatty oils. (The undissolved asphalt may be employed for colouring a fresh quantity of ether, in which substance it is not very soluble.)

442. Vesuvianin Celloidin Brown Injection.[2]—2. Make a concentrated solution of Vesuvianin in absolute alcohol and dissolve celloidin in it. (This colour is not fast.)

443. Opaque-Blue Celloidin Injection.[3]—3. Dissolve celloidin in equal parts of absolute alcohol and ether, and add pulverised Berlin-blue.

444. Opaque-Red Celloidin Injection.[4]—4. Proceed as above (3), taking pulverised cinnabar instead of Berlin-blue. The two last pigments should be rubbed up in a mortar with a little absolute alcohol and the paste added to the celloidin-mass. Be careful not to take more pigment than is absolutely necessary, or the injection will become brittle. To filter (if this be thought necessary), strain the mass through flannel wetted with ether. Syringes must be free from grease, which would render the mass brittle. The nozzles to be filled with ether. Inject quickly, as the mass soon sets on contact with watery tissues. Clean syringes and nozzles with ether.

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[1] 'Arch. Anat. u. Phys.,' 1882 (Anat. abth.), p. 201.

[2] Ibid. [3] Ibid. [4] Ibid.

Corrosion of the preparations.—The injected organs are thrown into unrectified hydrochloric acid, where they remain (the acid being changed from time to time if necessary) until all the tissues are destroyed. Wash under a slow stream of water from a tap furnished with an india-rubber tube. Leave for some weeks in water, rinse, and put up in glycerin, or a mixture of glycerin, alcohol, and water in equal volumes.

445. Natural Injections (*Robin's method*[1]).—To make preparations of naturally-injected organs, throw them into a liquid composed of:

Water	100 grammes.
Liquid perchloride of iron .	10 ,,

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[1] 'Traité,' p. 6.

CHAPTER XXXI.

MACERATIONS AND CORROSIONS.

446. It is sometimes necessary, in order to obtain a complete knowledge of the forms of the elements of a tissue, that the elements be artificially separated from their place in the tissue and separately studied after they have been isolated both from neighbouring elements and from any interstitial cement-substances that may be present in the tissue. Simple teasing with needles is often insufficient to effect the desired isolation, as the cement-substances are often tougher than the elements themselves, so that the latter are torn and destroyed in the process. In this case, recourse must be had to maceration processes, by which is here meant treatment with media which have the property of dissolving or at least softening the cement-substances or the elements of the tissue that it is not wished to study, whilst preserving the forms of those it is desired to isolate. When this softening has been effected the isolation is completed by teasing, or by agitation with liquid in a test-tube, or by the method of tapping, which last gives in many cases (many epithelia for instance) admirable results which could not be attained in any other way. The macerated tissue is placed on a slide and covered with a thin-glass cover supported at the corners on four little feet made of pellets of soft wax. By tapping the cover with a needle it is now gradually pressed down, whilst at the same time the cells of the tissue are segregated by the repeated shocks. When the segregation has proceeded far enough, mounting medium may be added, and the mount closed.

The student will do well not to neglect this simple method, which is one that it is most important to be acquainted with.

447. Iodised Serum.—Iodised serum was first recommended by Max Schultze ('Virchow's Archiv,' xxx, 1864, p. 263). I take the following instructions concerning it from Ranvier ('Traité,' p. 76).

The only serum that gives really good results is the amniotic liquid of mammals. A gravid uterus of a sheep or cow having been obtained (in large slaughter-houses such can be obtained without difficulty), an incision is made through the wall of the uterus and the fœtal membranes. A jet of serum issues from the incision, and is caught in a flask prepared for the purpose. Flakes of iodine are then added, and the flask is frequently agitated for some days. Two points should be noted. A perfectly fresh amnios must be taken; for the merest incipience of putrefaction will spoil the preparation. The flask should have a wide bottom, so that the serum may form only a shallow layer in it; otherwise the upper layers will not be sufficiently exposed to the action of the iodine.

Another method is as follows:—Serum is mixed with a large proportion of tincture of iodine; the precipitate of iodine that forms is removed by filtration, and there remains a strong solution of iodine in serum. This should be kept in stock, and a little of it added every two or three days to the serum that is intended for use.

Ranvier explains that at the outset serum dissolves very little iodine; but if an excess of iodine be kept constantly present in the solution, it will be found that after two or three weeks iodides are formed, and allow fresh quantities of iodine to dissolve; so that after one or two months a very strongly iodised serum is obtained. It should be dark brown. Such a solution is the most fitting for the purpose of iodising fresh serum in the manner directed above, and for making the different strengths of iodised serum that are required for different purposes. In general, for maceration purposes, a serum of a pale brown colour should be employed.

The manner of employing iodised serum for maceration is as follows:—A piece of tissue smaller than a pea must be taken, and placed in 4 or 5 c.c. of weakly iodised serum in a well-closed vessel. After one day's soaking the maceration is generally sufficient, and the preparation may be completed by teasing or pressing out, as indicated above; if not, the soaking must be continued, fresh iodine being added as often as the serum becomes pale by the absorption of the iodine by the tissues. By taking this precaution, the maceration may be prolonged for several weeks.

It is obvious that these methods are intended to be applied to the preparation of *fresh* tissues, the iodine playing the part of a fixing agent in regard to protoplasm, which it slightly hardens.

448. Artificial Iodised Serum (*Frey's formula*[1]):

Distilled water	135 grammes.
White of egg	15 "
Sodium chloride	0·20 "
Mix, filter, and add—	
Tincture of iodine . . .	3 "

There is formed a precipitate, which is removed by filtering through flannel; and a little iodine is added to the filtrate.

Ranvier states that he has been unable to obtain good results by this method.

449. Alcohol.—Ranvier employs one-third alcohol (1 part of 36° alcohol to 2 parts of water). Epithelia will macerate well in this in twenty-four hours. Ranvier finds that this mixture macerates more rapidly than iodised serum.

Other strengths of alcohol may be used, either stronger (equal parts of alcohol and water) or weaker (¼ alcohol, for isolation of the nerve-fibres of the retina, for instance, Thin).

450. Sodium Chloride and Alcohol (*Moleschott and Piso*

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[1] 'Le Microscope,' p. 131. Ranvier, 'Traité,' p. 77.

Borme's formula [1]).—Ten per cent. solution of sodium chloride, 5 volumes; absolute alcohol, 1 volume.

For vibratile epithelium, Ranvier finds the mixture inferior to one third alcohol.

451. Chromic Acid.—Generally employed of a strength of about 0·02 per cent. Specially useful for nerve tissues and smooth muscle. Twenty-four hours' maceration will suffice for nerve tissue. About 10 c.c. of the solution should be taken for a cube of 5 mm. of the tissue (Ranvier).

452. Bichromate of Potash.—0·2 per cent.

453. Müller's Solution and Saliva.—*See* below, No. 462.

453a. Permanganate of Potash—Has an action similar to that of osmic acid. Is recommended, either alone, or combined with alum, as the best dissociating agent for the fibres of the cornea (Rollett, 'Stricker's Handbuch,' p. 1108).

454. Osmic Acid.—0·1 per cent., for from a few minutes to a fortnight (cortex of cerebrum, Rindfleisch). May be followed by maceration in glycerin.

455. Osmic and Acetic Acid (*the Hertwigs' formula* [2]):

0·05 per cent. osmic acid . . . 1 part.
0·2 ,, acetic acid . . . ,,

Medusæ are to be treated with this mixture for two or three minutes, according to size, and then washed in repeated changes of 0·1 per cent. acetic acid, until all traces of free osmic acid are removed. They then remain for a day in 0·1 per cent. acetic acid, are washed in water, stained in Beale's carmine (in order to prevent the osmium from over-blackening and to assist the maceration), and are preserved in glycerin.

For *Actiniæ*,[3] the osmic acid is taken weaker, 0·04 per cent.;

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[1] Moleschott's 'Untersuchungen zur Naturlehre,' xi, pp. 99-107. Ranvier, 'Traité,' p. 242.

[2] 'Das Nervensystem u. die Sinnesorgane der Medusen,' Leipzig, 1878, p. 4.

[3] 'Jen. Zeitschr.,' xiii (1879), p. 457. 'Journ. Roy. Mic. Soc.,' iii (1880), p. 441, and (N.S.), iii (1883), p. 732.

both the solutions are made with sea-water; and the washing out is done with 0·2 per cent. acetic acid. If the maceration is complete, stain with picro-carmine; if not, with Beale's carmine.

456. Nitric Acid.—Most useful for the maceration of muscle. The strength used is 20 per cent. After twenty-four hours' maceration in this, isolated muscle-fibres may generally be obtained by shaking the tissue with water in a test-tube.

457. Nitric Acid and Chlorate of Potash (*Kühne's method*[1]).—Chlorate of potash is mixed, in a watch-glass, with four times its volume of nitric acid. A piece of muscle is buried in the mixture for half an hour, and then agitated with water in a test-tube, by which means it entirely breaks up into isolated fibres.

458. Sulphuric Acid.[2]—Sulphuric acid has been employed by Max Schultze for isolating the fibres of the crystalline.

Macerate for twenty-four hours in 30 grammes of water, to which are added 4 to 5 drops of concentrated sulphuric acid. Agitate.

Odenius found very dilute sulphuric acid to be the best reagent for the study of nerve-endings in tactile hairs. He macerated hair-follicles for from eight to fourteen days in a solution of from 3 to 4 grains of "English sulphuric acid" to the ounce of water.

Hot concentrated sulphuric acid serves to dissociate horny epidermic structures (horn, hair, nails).

459. Oxalic Acid.—Maceration for many days in concentrated solution of oxalic acid has been found useful in the study of nerve-endings.

460. Solution of Potash or Soda.[3]—These solutions must be employed *strong*, 35 to 50 per cent. (Moleschott); so employed they do not greatly alter the forms of cells, whilst weak solutions destroy all the elements. (Weak solutions may, however, be employed for dissociating the cells of epidermis, hairs, and nails.) The strong solutions may be employed by simply treating the tissues with them on the slide. It should be remembered that preparations obtained by means of these alkalies cannot be permanently preserved.

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[1] 'Ueber die peripherischen Endorgane, &c.,' 1862. Ranvier, 'Traité,' p. 79.

[2] Ranvier, 'Traité,' p. 78.

[3] Ibid.

461. Sulphocyanides of Ammonium and Potassium (*Stirling's method*[1]).—Ten per cent. solution of either of these salts is an admirable dissociating medium for epithelium. Macerate small pieces for twenty-four to forty-eight hours, stain with fuchsin, eosin, or picro-carmine.

If a crystalline be macerated as above its fibres become beaded or moniliform.

462. Saliva, Artificial (for embryology of nerve and muscle) (*Calberla's formulæ*[2]).—After having made trial of various different macerating agents, with the object of obtaining isolation of the developing muscle and nerve of embryos of *Amphibia* and *Ophidia*, Calberla found that the best results were obtained by means of Czerny's mixture of saliva and solutio Mülleri. This led him to imagine an artificial saliva, which on trial gave results as good as those obtained by natural saliva, or even better.

First formula.

Potassium chloride	1·8
Sodium chloride	1·2
Phosphate of soda	0·8
Calcium chloride	0·4
Sulphate of magnesia . . .	0·4
Sodium rhodanide	0·1
Carbonate of lime	0·4
Phosphate of lime	0·2
	5·3

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[1] 'Journ. Anat. and Phys.,' xvii (1883), p. 208.

[2] 'Arch. Mik. Anat.,' xi (1875), p. 449.

which are dissolved in 556 c.c. water. Carbonic acid is then passed through the solution to saturation. Two volumes of the solution are mixed with 2 vols. water and 1 vol. solutio Mülleri, and the embryos are macerated in the mixture for one or two days. Equally good results were obtained by the use of the following simpler solution:

463. Saliva, Artificial (*Calberla's second formula*):

Calcium chloride	0·4
Sodium chloride	0·3
Phosphate of soda	0·2
Calcium chloride	0·2
	1·1

This is dissolved in 100 parts of water, saturated with carbonic acid, and the solution combined with water and solutio Mülleri in the proportions given above in the first formula.

In either case, the Müller's solution may be replaced by a 2½ per cent. solution of chromate of ammonia. The best results were obtained when the solutions were saturated with the CO_2 just before using.

The tissues are isolated by teasing and shaking, and specimens mounted in concentrated acetate of potash.

464. Corrosion (*Altmann's methods*[1]).—The applicability of the principle of corrosion to histology is found in the fact that whilst almost all animal tissues are very quickly destroyed by eau de Javelle, yet fats, and particularly fats hardened by osmic acid, withstand its action for a long time. If then you introduce some fat or other into a tissue, harden it with osmic acid and corrode the tissue with eau de Javelle, you will obtain a mould, in osmium-blackened and hardened fat, of the spaces you had filled with the fat introduced.

The fat used by Altmann is olive oil; he also uses the fat-like resin, castor oil. The corroding agent is eau de Javelle (*Aqua Javelli*), a well-known industrial bleaching and cleansing agent. It consists of a solution of hypochlorite of soda or potash, containing a certain excess of chlorine.

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[1] 'Arch. Mik. Anat.,' xvi (1879), p. 471, *et seq.*

Injection.—The fat may be introduced into the tissues either by injection or by impregnation. In the first case, which naturally applies to the study of vessels, we proceed as follows:—Olive oil is carefully injected; and the injected piece of tissue, if thin enough, is thrown at once into 1 per cent. osmic acid; but thick parenchymatous organs must first be cut into sections thin enough to ensure the penetration of all parts by the osmic acid. This is done by the freezing process, in order to guard against the escape of the oil from cut surfaces (Altmann uses for freezing, pounded ice and nitrate of soda). The preparations remain about twenty-four hours in the osmic acid. They are then immersed in Aqua Javelli (in small glass vessels, in order that the progress of the corrosion may be readily followed). From a few minutes to several hours are required to complete the corrosion. The sections are carefully watched, and at the right moment carefully floated out by means of a bent strip of platinum, dried off at the edges with blotting-paper, and brought into glycerin on a slide. They are there washed by dropping glycerin on to them, and may then be permanently mounted.

If it be desired, the eau de Javelle may be diluted with water, and the immersion prolonged, which enables one to control the process of corrosion more exactly.

The process is very useful for the study of the vessels of deeply-pigmented tissues, such as the choroid and the iris.

Frogs or tritons may be injected from the aorta with olive oil, the eye extracted and thrown into osmic acid; after a few hours the bulb may be opened and replaced in the osmic acid, and when the reaction is complete, the choroid or the iris may be dissected out, corroded, and mounted as above indicated.

For the purposes of interstitial injection the method proves particularly valuable. The skin of the frog may be interstitially injected from the aorta by using sufficient pressure to force the oil out of the capillaries. On preparing a portion of the skin by corrosion it will then be found that the lymphatics are more or less completely injected, and successful preparations further demonstrate the system of fine tortuous canals, leading from the blood-vessels to the lymphatics, described by Arnold. The best preparations are those afforded by the skin of the inner surface of the hind leg, and it is desirable to set up a state of œdema before injecting. A ligature is placed between the abdomen and the thigh just tight enough to cause hindrance to the return-flow of the lymph and venous blood, without suppressing the arterial circulation. In two or three days the skin will be found sufficiently hyperæmic. The ligature is then removed, the apex of the ventricle cut off, and the frog thoroughly bled, one aorta tied and oil injected from the other (the veins not being tied) until pure oil alone flows from the open ventricle; the veins are then tied and the injection continued. (For further details and for the precautions it is desirable to take, as well as for the description of the results, I refer the reader to the interesting paper from which I am abstracting.)

Good preparations of this sort may also be obtained from the mesentery of the frog and of *Salamandra maculata* (these need not be corroded, as the membranes are thin enough to be transparent); and from *Triton tæniatus*, by corrosions of the end of the tail.

The corneal corpuscles of *Triton cristatus* may sometimes be injected from the aorta, and in the rabbit excellent preparations may be obtained from the cranial periosteum, the oil being injected (by the pressure obtained from a flask of oil raised three metres high) into the carotid artery. (See the details as above.)

465. Impregnation.—The principle of this process is that the fatty substance is mixed with some medium that will cause it to change places, by diffusion, with the watery components of the tissues. If to one volume of olive oil be added about half a volume of absolute alcohol and a like quantity of ether (the exact proportion of ether being that which gives a clear mixture by agitation), a mixture is obtained which has the property of taking up *small* quantities of water without undergoing separation into its constituent parts. If *small* portions of tissue be immersed in such a liquid, all those constituents of the tissue that are soluble in the oil-mixture (namely, first of all its water, then fatty and nervous matters), will be taken up by it, and the oil-mixture will take their place in the tissue. If now the latter be thrown into water the alcohol and ether will be taken up by the water, and the oil thrown down in the tissue. This is then hardened and blackened with osmic acid and corroded with Aqua Javelli.

It must be noticed that this method of impregnation differs in principle from the ordinary methods of impregnation with metallic salts, inasmuch as these depend on chemical affinities, whilst the oil-impregnation depends on purely physical processes of diffusion.

Second formula (*Castor-oil and alcohol*).—Instead of the above-given oil-mixture, it is sometimes advantageous to use the following: 2 parts of castor-oil are dissolved in 1 part of alcohol. (Castor-oil being soluble in alcohol, it is unnecessary to add ether.)

Fresh tissues should always be employed for impregnation. *Small* portions should be immersed for from five to eight days in a large quantity of the oil-mixture, then thrown into water in order to precipitate the oil, and finally brought for twenty-four hours into a 1 per cent. solution of osmic acid, and finally corroded and mounted in glycerin.

The author describes (l. c.) the results he obtained in the study of dark-bordered nerve-fibres, muscle-fibres, cartilage, epithelia, cornea, choroid, retina. He appears to be justified in claiming for the method that it is likely to be of much use in certain special researches. I recommend the reader to carefully study the article, which does not well bear abstracting.

The proportions of the oil-mixture may be varied within certain limits if desired. Those quoted are, however, recommended as having given the best results. If it happen that too large portions of tissue have been taken, so that the mixture becomes cloudy, a little ether may be added to restore the balance, but care must be taken not to add so much ether as seriously to change the proportional composition of the mixture.

466. Eau de Javelle (Hypochlorite of Potassium) (*Noll's method*[1]).—Noll remarks that the usual method of preparing the skeleton of siliceous sponges and similar structures by corroding away the soft parts by means of caustic potash has many disadvantages, of which a principal one is that the spicula are not preserved in their normal positions. He therefore proceeds as follows: A piece of sponge is brought on to a slide and treated with a few drops of eau de Javelle, in which it remains until all soft parts are dissolved. (With thin pieces this happens in twenty to thirty minutes.) The preparation is then cautiously treated with acetic acid, which removes all precipitates that may have formed, and treated with successive alcohols and oil of cloves, and finally mounted in balsam.

The same process is applicable to calcareous structures.

Eau de Javelle may also be used for clearing plant sections. After treatment for a quarter of an hour, everything is removed from such sections except the mere cell-walls; the sections are then washed with acetic acid and mounted in glycerin-jelly or Meyer's fluid (glycerin 1 part, water 2 parts, to 10 volumes of which mixture are added 1 volume of salicylic vinegar—1 per cent. solution of salicylic acid in pyroligneous acid).

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[1] 'Zool. Anzeig.,' 122 (1882), p. 528.

467. Caustic Potash, Caustic Soda, Nitric Acid.—Boiling, or long soaking in a strong solution of either of these is an efficient means of removing soft parts from skeletal structures (appendages of arthropods, spicula of sponges, &c.).

CHAPTER XXXII.

DECALCIFICATION, DESILICIFICATION, AND BLEACHING.

468. Decalcification of Bone.[1]—I take the following historical sketch from Busch's article "On the Technique of the Histology of Bone," l. c.

The most widely-used agent for decalcification is hydrochloric acid. Its action is rapid, even when very dilute, but it has the disadvantage of causing serious swelling of the tissues. To remedy this chromic acid may be combined with it, or alcohol may be added to it. Or a 3 per cent. solution of the acid may be taken and have dissolved in it 10 to 15 per cent. of common salt. Or (Waldeyer) to a $\frac{1}{1000}$ per cent. solution of chloride of palladium may be added $\frac{1}{10}$th of its volume of HCl.

Chromic acid is also much used, but has a very weak decalcifying action and a strong shrinking action on tissues. For this latter reason it can never be used in solutions of more than 1 per cent. strength, and for delicate structures much lower strengths must be taken.

Phosphoric acid has been recommended for young bones.

Acetic, lactic, and pyroligneous acid have considerable decalcifying power, but cause great swelling. Picric acid has a very slow action, and is only suitable for very small structures.

469. Nitric Acid (*Busch's formula*[1]).—To all other agents Busch prefers nitric acid, which causes no swelling and acts most efficaciously, whilst at the same time it does not injuriously attack tissue-elements.

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[1] 'Arch. Mik. Anat.,' xiv (1877), p. 481.

One volume of chemically pure nitric acid of sp. gr. 1·25 is diluted with 10 vols. water. It may be used of this strength for very large and tough bones; for young bones it may be diluted down to 1 per cent.

Fresh bones are first laid for three days in 95 per cent. alcohol; they are then placed in the nitric acid, *which is changed daily*, for eight or ten days. They must be *removed as soon as* the decalcification is complete, or else they will become stained yellow. When removed they are washed for one or two hours in running water and placed in 95 per cent. alcohol. This is changed after a few days for fresh alcohol.

Young and fœtal bones may be placed in the first instance in a mixture containing 1 per cent. bichromate of potash and $\frac{1}{10}$ per cent. chromic acid, and decalcified with nitric acid of 1 to 2 per cent., to which may be added a small quantity of chromic acid ($\frac{1}{10}$ per cent.) or chromate of potash (1 per cent.). By putting them afterwards into alcohol the well-known green stain is obtained.

Staining agents.—Sections of bone treated in the last-described manner are stained five or ten minutes in a weak aqueous solution of eosin. The ground-substance and small cells of cartilage remain colourless, the nuclei of the large cells are stained red, and so is periosteum, bone-tissue, and the cellular contents of the medullary spaces. Hæmatoxylin may be used in conjunction with eosin (before or after it), to obtain double-stains, which, however, are seldom successful.

Sections are dehydrated in absolute alcohol, and mounted (*without* clearing by oil of cloves or the like) in a benzol-solution of Canada balsam.

470. Chromic Acid is employed in strengths of from 0·1 per cent to 1 per cent., the maceration lasting two or three weeks (in the case of bone). It is better to take the acid weak at first, and increase the strength gradually.

471. Hydrochloric Acid may be taken of 50 per cent. strength, and then has a very rapid action (Ranvier).

472. Picric Acid should be taken saturated.

Picro-sulphuric acid should of course be avoided on account of the formation of gypsum.

473. Chromic and Nitric Acid.—Dissolve 15 gr. pure chromic acid in 7 oz. of distilled water, to which 30 minims of nitric acid are afterwards to be added. Macerate for three or four weeks, changing the fluid frequently (Marsh).

474. Glycerin. Alum-Carmine.—It should be remembered that these commonly-used reagents dissolve carbonate of lime; they must therefore be avoided in the preparation of structures containing calcareous elements that it is wished to preserve (calcareous sponges, echinodermata, &c.).

DESILICIFICATION.

475. Hydrofluoric Acid (*Mayer's method*[1]).—The objects from which it is desired to remove siliceous parts are brought in alcohol into a glass vessel coated internally with paraffin (otherwise the glass would be corroded by the acid). Hydrofluoric acid is then added drop by drop (the operator taking great care to avoid the fumes, which attack mucous membranes with great energy). A *Wagnerella borealis* may thus be completely desilicified in a few minutes. Small pieces of siliceous sponges will require a few hours or at most a day. The tissues do not suffer; and if they have been previously stained with acetic acid carmine, the stain does not suffer; at least this was so in the case of *Wagnerella*.

(As regards sponges, I would point out that if well imbedded, good sections may be made from them without previous removal of the spicula. The spicula appear to be cut; probably they break very sharply when touched by the knife. Knives are of course not improved by cutting such sections.)

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[1] 'Zool. Anz.' (1881), No. 97, p. 593.

476. Chloride of Calcium for Bleaching (*Paul Mayer's method*[1]).—This is a process imagined for the purpose of getting rid of the blackening that often occurs as a consequence of over-staining by osmic acid.

The specimens are put into alcohol (either of 70 or 90 per. cent). Crystals of chloride of calcium are added until the bottom of the vessel is covered with them. A few drops of concentrated hydrochloric acid are then added by means of a pipette, and mixed-in by shaking the vessel as soon as the green colour of the evolving chlorine has begun to show itself. Warm if necessary; but most objects, even large ones, may be bleached in half a day without the employment of heat. The tissues do not suffer.

Instead of hydrochloric acid, nitric acid may be used; in which case the bleaching agent is the freed oxygen, instead of chlorine.

The first method may be used for the purpose of removing pigment from the eyes of insects.

477. Chlorine Bleaching Process (*Marsh's method*[2]).—Marsh generates chlorine in a small bottle by treating crystals of chlorate of potash with strong HCl, and leads the gas (by means of a piece of glass tubing bent twice at right angles) to the bottom of a bottle containing the sections in water. (See a fig. of the apparatus in 'Journ. Roy. Mic. Soc.,' iii (1880), p. 854.)

478. Chlorine Solution (*Sargent's method*[3]).—Hydrochloric acid, 10 drops; chlorate of potash, $\frac{1}{2}$ dr.; water, 1 ounce. Soak for a day or two. Wash well.

This method is intended for "bleaching insects;" it will be seen that it is only applicable to the preparation of hard parts as soft tissues would be destroyed by the solution.

479. Chloride of Lime; Chlorinated Soda.—These and other bleaching solutions may of course be employed, but only in the preparation of hard parts, as soft tissues are destroyed by them.

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[1] 'Mitth. Zool. Stat. Neapel.,' ii (1881), p. 8.

[2] 'Section Cutting,' p. 89.

[3] 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 151.

480. Kreasote (*Pouchet's method*[1]).—I gather from the paper here quoted that most of the granular animal pigments are soluble in kreasote. Other solvents are mentioned in this paper ("On the Change of Colouration through Nervous Influence"), but this appears to be the only one capable of general histological application.

481. Nitric Acid.—Nitric acid has a similar action.

482. Oxygenated Water (*Pouchet's method*[2]).—Macerate in glycerin to which has been added a little oxygenated water (5 to 6 drops to a watch-glass of glycerin). (Oxygenated water may be procured from perfumers or hair-dressers, by whom it is sold as a hair dye under the name of "Auréoline," "Golden hair-wash," or the like.)

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[1] 'Journ. de l'Anat.,' 1876, pp. 8, *et seq.*

[2] M. Duval, 'Précis, &c.,' p. 234.

PART II.

SPECIAL CASES AND EXAMPLES.

482 a. It was originally my intention to take the student in this Second Part, through the entire field of Histology and Microscopic Zootomy, giving him detailed instructions for the examination of all structures that have hitherto been studied, and thus making him entirely independent of all help from a teacher. I have abandoned that idea, partly on account of the magnitude of the task, and partly because I no longer think that such a work is necessary. I think that the student who has mastered the principles of the methods set out in the first part of this work should hardly be in need of any help in the application of those methods, and I hope that the classifications I have adopted and the explanations I have given will suffice to make it easy to any student to master those principles.

Nothing more is wanted, I think, than that the application of general methods to very special cases, such as the study of cell-division, or the Microtomy of the human brain, should be discussed, and that some few hints be given as to the choice of a method in all cases in which the matter is not quite simple. In the following paragraphs I have discussed such special cases as appeared to require discussion, and have added numerous examples, and hope that although this second part is only a collection of such discussions and examples it may be found to have the usefulness of a formal treatise.

CHAPTER XXXIII.

CYTOLOGICAL METHODS.

483. Cell-division (*Peremeschko's methods*[1]).—The objects studied were the tissues of the tail of larvæ of *Triton cristatus.* The reagents used were: Gold chloride, ½ to ⅕ per cent.; osmic acid, ½ to ⅕ per cent.; silver nitrate, ½ per cent.; and absolute alcohol. The alcohol is most to be recommended. For staining, hæmatoxylin, fuchsin, and neutral solution of carmine were used. Tissues may be left for a quarter of an hour in absolute alcohol, stained with one of the above-mentioned stains and mounted in glycerin or dammar.

In the living animal the epithelial cells and nuclei (in the state of repose) are so transparent as to be invisible in the natural state. They may, however, be brought out by curarising the larva; or still better, by placing the curarised larva for half an hour in 1 per cent. chloride of sodium solution. Normal larvæ may be used for the study of the active state of the nucleus, but much time is saved by using curare.

Curare.—Dissolve 1 part of curare in 100 parts water, and add 100 parts of glycerin. Of this mixture add from 5 to 10 drops (according to the size of the larva), or even more for large larvæ, to a watch-glassful of water. From half to one hour of immersion is necessary for curarisation. The larvæ need not be left in the solution until they become quite motionless; as soon as their movements have become slow they may be taken out and placed on a slide with blotting-paper.

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[1] 'Arch. Mik. Anat.,' xvi (1879), p. 437.

If they be replaced in water they return to the normal state in eight or ten hours and may be re-curarised several times.

Etherisation.—Three per cent. alcohol, or 3 per cent. ether, may be used in a similar way. These reagents cause no obstruction to the processes of cell-division, and are useful, but their action as anæsthetics is inconstant.

Indifferent media.—One per cent. salt solution, iodised serum, syrup, cold water (+ 1° C.), and warm water (35—40° C.). The tail may be excised from the living animal and studied for a long time in these media.

484. Methods for the Study of Cells (*Flemming's methods*[1]).—The best subject for these studies is Salamandra. The adult offers for study the thin transparent bladder; in the larva the gills and caudal "fin" may be studied in the living state. The gills are difficult to fix in position for observation, and are obscured by pigment. In the fin, there is always a spot, near to the hind-limbs, that is free from pigment; and on lightly-coloured larvæ other such spots may be found, on the ventral half of the fin and on the lateral line. On a flat-finned larva it is possible to study these spots with a Hartnack's No. 12 imm.

The larva may be fixed in a suitable cell; or wrapped in moist blotting-paper; or may be curarised; or the tail may be excised. (It is preferable to cut through the larva close in *front* of the hind-limbs).

A favorable object for *preparation* is found in the gill-*plates*, delicate laminæ that are to be found attached to the gill-cartilages on the mouth side.

Larvæ may be bred from adults kept in confinement, and supplied with a vessel of water, in which they will place the larvæ of their own accord. In May, gravid females may be killed and the larvæ extracted. The larvæ must be kept in frequently changed water, and fed every day or two. Aquatic worms may be used for feeding them; viz. *Tubifex rivulorum.*

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[1] Ibid., p. 304, *et seq.*

So-called "indifferent" liquids must not be believed to be without action on nuclei. Iodised serum, salt-solution, serum, aqueous humour, lymph, better deserve the name of weak hardening agents.

Osmic acid ($\frac{1}{10}$th to 2 per cent.) preserves the form of the entire cell, but swells the nuclei and rounds off nucleoli. It renders the nuclear reticulum undiscernible. Picric acid, either concentrated or dilute, and chromic acid, 0·1 to 0·5 per cent. are the best fixing agents for nuclei. Stronger chromic-acid solutions cause shrinking. Neither of these reagents is harmless as regards the nuclei of red blood-corpuscles. The salts of picric acid (potash-, soda-, and baryta-salts) are most harmful. Weak acetic, hydrochloric, or nitric acid combined with clearing in glycerin, and staining, may be useful for bringing out reticula and nucleoli. Chloride of gold preserves the forms well, but generally leaves the nuclear structures unstained. Nitrate of silver is hopelessly uncontrollable in its action. Alcohol has much the effect of chromic acid, but often causes a much greater shrinking of the nuclei. Bichromate of potash and chromate of ammonia bring out very sharply the appearance of a reticulum, but these appearances cannot be accepted as true (l. c., p. 334 ff.). Karyokinetic figures are quite spoiled by them.

The best results were obtained with chromic-acid preparations well washed out and treated for some days with absolute alcohol before staining with anilin colours (safranin, rose de naphthalin, &c.). Almost equally good results were obtained by means of hæmatoxylin, which should be "of middle strength," and allowed to act for at most half an hour, in the case of chromic-acid preparations; whilst in the case of picric-acid preparations the hæmatoxylin should be "as weak as possible" and be allowed to act for a long time. Mount in glycerin or in balsam; water is a still better medium for study of the preparations on account of the stronger images it affords. Picro-carmine applied to *fresh* objects causes swelling of the nuclei; but used with picric-acid specimens it gives true appearances.

Cell-division.—For the special study of the phenomena of cell-*division*, the best object is the above-mentioned gill-plates of the larva of salamandra. Picric acid in saturated solution is the best reagent for preserving the karyokinetic figures. (Care must be taken not to let the preparations be invaded by mould.) Stain, if required, with hæmatoxylin (in weak solution) or carmine. Chromic acid and chloride of gold may also be used.

485. Fixing Agents for Study of Cell-anatomy (*Flemming's methods*[1]).—Picric and chromic acid (to be followed by hæmatoxylin- or anilin-staining) are to be preferred to alcohol and other agents for the study of the cells of *Vertebrates*. Shrinking and distortion of the nuclear figures (and, with picric acid, swellings of them) are to be expected, but other agents have the same defect to a much greater degree; alcohol especially causes *entanglement* of the filaments. Acetic acid does the same, and causes swelling besides.

"*Those who seek to study cell-division by means of bichromate of potash or other chromic salts are hopelessly in the wrong road.*"[2] And this, because of the injurious action of the bichromate, *not* on the body of the cell, which it preserves well, but on the karyokinetic figures. Osmium is useless, because it does not sufficiently stain the nuclear figures, and prevents them from staining well with other reagents. In the case of epithelium, thin sections should be made through tissues hardened in chromic or picric acid, stained with hæmatoxylin or anilin, and cleared. This is better than to attempt to isolate the cells. Picric-acid solutions *must never be weak.*

For staining he recommends:

1. Bismarck brown (Mayzel's formula, No. 155).

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[1] 'Arch. Mik. Anat.,' xviii (1880), p. 250.

[2] L. c., p. 352.

2. Alum-carmine (Partsch's formula, 'Arch. Mik. Anat.,' xiv (1877), p. 180).

3. Picro-carmine (often causes swellings, especially when not quite neutral).

4. Anilin.

5. Hæmatoxylin. (The two last are the most troublesome, but give the best results. With hæmatoxylin it is best to use very dilute solutions, and to stain very slowly. For the nuclei of vegetal cells any of the usual stains may be used with good results; hæmatoxylin is apt to stain too intensely.)

486.[1] These processes have since been further worked out, and Flemming now (1882) adds the following instructions:

All fixing agents alter cell-structures if they are allowed to act too long; it is always best to examine specimens after not more than half an hour's immersion.

In opposition to Henle ('Arch. Mik. Anat.,' xx Bd., 4 Heft), Flemming maintains his above-quoted position "that those who work at nuclear figures with chromic salts are hopelessly in the wrong road." Chromic salts are excellent reagents for general histological work, but not for nuclear structures. They dissolve nucleoli, destroy nuclear networks, and swell-up and distort karyokinetic figures to such a degree that the appearances obtained from them are merely unnatural caricatures of the true structure.

Chromic acid gives equally good results in all strengths from $\frac{1}{5}$ to $\frac{1}{2}$ per cent.; for certain plant-structures it may be taken of 1 per cent.

Picric acid may be taken either concentrated or weaker. Acetic or formic acid should not be stronger than 1 per cent.

Altmann's nitric-acid method (formula No. 28) is excellent for the purpose of hunting for cell-divisions in tissues; but the minute structure of the figures is not so well preserved as it is by means of chromic or picric acid. The same must be said of Kleinenberg's picro-sulphuric acid method.

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[1] 'Zellsubstanz, &c.,' p. 379, *et seq. et passim.*

The best fixing agent in general is the chromo-aceto-osmic acid mixture (No. 14). Attempts to omit the chromic acid did not give good results. The omission of acetic acid (as in Max Flesch's formula, No. 13) causes the figures to be far less sharply brought out. The presence of acetic or formic acid in all osmium solutions is favorable to the precision of subsequent staining with hæmatoxylin, picro-carmine, or gentian-violet. But mixtures of osmic and acetic acid without chromic acid (Eimer) do not give such good results as the chromo-aceto-osmic acid mixture. Mixtures of picric acid with osmic acid or with osmic and acetic acid (proportions of the latter as in the chromo-acetic osmic mixture (14), but of picric acid about 50 per cent.) fix quite as well as the chromic mixtures, but precise staining is even more difficult than with pure osmic-acid preparations. Flemming concludes that the beneficial effects of the osmium in all these mixtures are to be ascribed to the instantaneous rapidity with which it kills, the function of the other acids of the mixture being to render the structures distinctly visible.

Mixtures containing osmic acid should therefore be employed whenever it is desired to fix the chromatic figures as faithfully as possible; whilst pure chromic acid should be taken whenever very sharp staining is the more important point.

For the study of the achromatic figures he recommends the chromo-acetic acid mixture No. 15, followed by staining in hæmatoxylin (anilins do not give so good results for this purpose).

For the study of polar corpuscles he recommends the osmium mixtures, or pure chromic acid followed by staining with gentian-violet.

For pure nuclear stains he continues to prefer the Böttcher-Hermann method (No. 136). The stains recommended most are *safranin*, *rose de naphthalin*, *dahlia*, and *gentian-violet*. For *fresh* preparations mixtures of *methyl-green* and *acetic* *acid* are very useful, especially for the purpose of staining under the cover-glass; but the stain is not permanent. *Bismarck brown and acetic acid* is also useful, but here too the stain is not permanent. *Gentian-violet* with *acetic acid* gives a stain that keeps much better (in glycerin). Schneider's *acetic-acid carmine* (No. 79) is very much to be recommended for fresh preparations.

The stains that keep best in dammar are safranin and naphthalin; hæmatoxylin and gentian fade after a year or so, the former in dammar as in glycerin.

Resinous turpentine is recommended for mounting objects that are liable to shrink in passing from clove oil into dammar.

487. Karyokinesis (*Pfitzner's methods*[1]).—Larvæ of *Salamandra maculata* of 22 mm. length were fixed with picric acid, washed out, and preserved in alcohol. Some of them were then stained in ammonia-carmine, some in hæmatoxylin (Kleinenberg's formula), some left unstained; all were imbedded in Calberla's albumen-mass, and put for some months into alcohol; after which sections were cut. The ammonia-carmine stains were most beautifully sharp, probably on account of the long immersion in alcohol. The unstained specimens were stained after cutting with safranin. Pfitzner insists on the necessity of having very thin sections; in the case of small-celled animals they should not be more than 7 to 10 μ. He uses the Thoma microtome, with which he can obtain sections of not more than 3 μ in thickness. He recommends the use of homogeneous-immersion objectives, homogeneous-immersion illuminators, and monochromatic light. The latter should be of a colour complementary to that of the nuclear stain, or nearly so. Flemming also in his last researches (quoted above) used oil-immersion objectives and the *full aperture* of Abbe's illuminator so as to work with the pure "absorption-image."

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[1] 'Arch. Mik. Anat.,' xx (1881), p. 135.

488. Karyokinesis (*Uskoff's methods*[1]).—Fresh embryos (for instance) are fixed in 5 per cent. nitric acid for ten to thirty minutes, rinsed in dilute alum-solution, and put for twelve to twenty-four hours into weak alcohol. Stain in Grenacher's alum-carmine for twenty-four hours (or forty-eight if the object be a large one), put into common alcohol until wanted, imbed in spermaceti, mount sections in glycerin or balsam.

489. Karyokinesis (*Strasburger's methods*[2]).—1. Fix with alcohol (absolute); stain with safranin (a solution of safranin in absolute alcohol, to which is added one volume of water) twelve to twenty-four hours, wash out in absolute alcohol till no more colour comes away; clear in oil of cloves, mount in cold dammar (a perfectly precise nuclear stain, permanent).

2. Fix with 1 per cent. acetic acid to which a little methyl-green is added. A nuclear stain, but does not last more than a few hours.

3. Fix with 50 per cent. nitric acid, wash, stain with methyl-green.

490. Division of Ovum (Echinodermata) (*Flemming's method*[3]).—The ova are stained on the slide by adding the stain at the edge of the cover. Safranin or other nitro- or anilin-colours may be used. As soon as the entire ovum is of a dark colour the stain is drawn off with blotting paper, and acetic acid of 1 per cent. added. Schneider's acetic carmine ('Zool. Anzeig.,' 1880) (*see* Formula No. 79) is very convenient, and gives good results. (For the details of the manipulation by which these reagents are added and drawn off on the slide it is well to consult the article quoted, p. 6.)

Another good method is as follows:—Segmenting ova are treated with a mixture of 40 to 50 parts of concentrated ic acid with 60 to 50 parts water. Wash with water until he yellow stain of the nitric acid has disappeared; stain ı Schneider's acetic carmine, and mount in glycerin. (The ›arations cannot be said to be permanent, as after a time stain darkens in such a way as to render the nuclear res unrecognisable.)

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[1] Ibid., xxi (1882), p. 292.

[2] Ibid., p. 477.

[3] Ibid., xx (1881), p. 3.

CHAPTER XXXIV.

EMBRYOLOGICAL METHODS.

491. Embryology in General.—The "general method" described in Section 2 of Part 1 is pre-eminently an embryological method. It would be superfluous to give here a detailed chapter on embryological methods, as such a chapter could do little more than repeat the description of the general method. I shall therefore confine myself to the quotation of one or two examples that may be helpful to the beginner.

492. Bufo cinereus, Embryology of (*Oellacher's method*[1]).—Embryos are hardened in CrO_3 until the pigment is quite removed, washed out with alcohol followed by glycerin for many hours, and imbedded in wax and oil; or they are removed from the CrO_3 *before* the pigment has quite disappeared, and passed through alcohol into turpentine.

493. Eye of Vertebrate Embryos (*Mihalkovic's methods*[2]).—The embryos are placed for twenty-four hours in $\frac{1}{5}$—$\frac{1}{6}$ per cent. chromic acid, then for some weeks in solutio Mülleri. Stain *in toto* with Thiersch's carmine, and place for twenty-four to forty-eight hours in alcohol. Bring them (in order to remove the alcohol) for a few minutes into distilled water, then into glycerin-jelly (glycerin 1 part, gelatin 1 part), which is kept liquid over a water-bath for half an hour in order that the jelly may fill the cavities of the organisms. They are then imbedded, by means of glycerin-jelly, in a hole made in a piece of liver, and the whole placed for two or three days to harden in strong alcohol. Sections are mounted in glycerin without removing the attached portions of the jelly.

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[1] 'Arch. Mik. Anat.,' vii (1872), p. 158.

[2] Ibid., xi (1875), p. 386.

494. Mounting Chick Embryos Whole.[1]—This is easily done by excising a blastoderm under salt-solution, floating it on to a slide, and there fixing, staining, dehydrating, and finally mounting it. It is, however, necessary to take special precautions to prevent the borders of the blastoderm from curling-up during the hardening process. This may be prevented by means of the method of demi-desiccation. The fresh blastoderm is allowed to begin to dry until its edges adhere to the slide; it may then safely be brought into the fixing solution.

Fix with 0·1 to 0·5 per cent. chromic acid (twenty-four hours), and wash-out with water followed by alcohol, or with osmic acid. In this case, after washing out, the preparation should be treated for some hours with some reagent capable of preventing the osmium from after-blackening (Müller's solution, chromic acid 0·5 per cent., Merkel's solution). Stain as desired, dehydrate, clear with clove oil, and mount in balsam.

495. Sections of Chick Embryos.[2]—For embryos of thirty-six to forty-eight hours Foster and Balfour recommend the following methods:

The embryo is to be removed under the surface of salt-solution (0·75 per cent.) kept at a temperature of 38° (the shell being broken into above the air-chamber, and removed piece by piece with forceps until the embryo is exposed). The embryo should then be floated on to a slide, fixed in place there by demi-desiccation as recommended above, and brought into the fixing solution. Foster and Balfour recommend chromic acid of 0·1 per cent. for twenty-four hours, followed by 0·3 per cent. for twenty-four more; after which 70 per cent. alcohol one day, and 90 per cent. alcohol two days, followed by absolute alcohol. They also recommend Kleinenberg's picro-sulphuric acid, five hours, followed by alcohol in the usual way. If osmic acid be taken the embryo should be put into a 0·5 per cent. solution for two and a half hours in the dark, and then washed out completely by means of several changes of absolute alcohol. In this case the embryo should be imbedded after twenty-four hours at the most, otherwise it may become too brittle to afford good sections.

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[1] Foster and Balfour, 'Éléments d'Embryologie,' Appendice.

[2] L. c.

For staining they recommend Beale's or any alcoholic carmine, or Kleinenberg's hæmatoxylin.

For imbedding they recommend paraffin and axunge (No. 228), or wax and oil (No. 227), or spermaceti (Nos. 237-8). They prepare the object for the paraffin-bath by soaking in kreasote, for the wax- and oil-bath by soaking in clove oil, and for the spermaceti-bath by soaking in bergamot oil. They remove the spermaceti mass from the sections by means of a mixture of 4 parts of turpentine to one of kreasote. The knife should in this case be wetted with olive oil; in the other cases, with kreasote or clove oil.

These methods may still be found useful, but must be said to have become somewhat antiquated since the working out of the refined methods of imbedding in paraffin, in celloidin, in egg-mass, or in soap masses, that have been described in the chapter on IMBEDDING METHODS, and of mounting in series that have been described in the chapter on SERIAL SECTION METHODS.

496. Urodela. Development of Cranium (*Stöhr's method*[1]). —Larvæ of Urodela are hardened in chromic or picric acid (Foster and Balfour's method, *supra*), followed by alcohol, (weak, strong, absolute). The smaller larvæ are then stained *in toto* in dilute ammonia carmine. Large larvæ are decalcified before staining by means of saturated picric-acid solution. (The head of a full-grown Triton may require months for complete decalcification.) They are taken from the picric acid direct into a *strong* ammonia-carmine solution. After eighteen to twenty-four hours they are washed out through several changes of water. Six to ten hours are required for the washing out. They are then passed through alcohol of gradually increasing strength.

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[1] 'Zeit. Wiss. Zool.,' xxxiii (1880), p. 479.

Bismarck brown may advantageously be used instead of carmine. The objects may be decalcified in nitric acid of 1 to 2 per cent. (Busch's formula, 'Arch. Mik. Anat.,' xiv, No. 468), washed out, and brought into the dye, which is prepared by diluting a saturated solution of Bismarck brown with 50 parts of absolute alcohol. This stain has the property of staining the ground-substance of hyaline cartilage dark brown, so that the cartilaginous parts of the skeleton can be recognised in sections even with the naked eye.

For imbedding, Kleinenberg's mixture of spermaceti and castor-oil was tried, but abandoned on account of the necessity of cutting "wet," which frequently disturbs the natural arrangement of the parts, by floating them away from their true positions. Braun's method of cutting "dry" is to be preferred. The objects are passed from the absolute alcohol into turpentine (for from six to twenty-four hours), and from this into a bath of concentrated solution of paraffin in turpentine (where they remain for another six to twenty-four hours); they are then imbedded in a mixture of 4 parts of paraffin with 1 of hog's lard. After the sections have been cut (with a dry knife), the imbedding-mass is to be dissolved out of them by means of a drop of turpentine. Mount in dammar.

497. Ovum of Fowl (*Koller's method*[1]).—Chromic acid, 0·1 per cent., twenty-four hours; ibid., 0·2 per cent., twenty-four hours; and so forth, with daily increments of 0·1 per cent. up to 0·5 per cent. When hard, remove the blastoderm together with a segment of the yolk. Water, twenty-four hours; weak ammonia-carmine, twelve to twenty-four hours; water, twenty-four hours; absolute alcohol, one to two days. Oil of cloves, a few minutes; imbed in wax and oil, and cut with turpentine.

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[1] 'Arch. Mik. Anat.,' xx (1881), p. 182.

498. Embryology of Lacerta (*Strahl's method*[1]).—Kleinenberg's liquor, followed by absolute alcohol and picro-carmine; the sections when cut were mounted in balsam.

499. Meroblastic Ova (*Kupffer's method*[2]).—The ova are opened and the albumen removed under osmic acid of $\frac{1}{10}$th per cent. The yolk is put for twenty-four hours into an ample quantity of $\frac{1}{3}$ per cent. chromic-acid solution; the blastoderm is removed, washed out in water, and put for three hours into Calberla's liquid (āā glycerin, water, and alcohol), and finally hardened in 90 per cent. alcohol.

They are stained with Böhn's neutral carmine (Formula No. 57) for twenty-four hours (or more, if of a greater thickness than 1 mm.), and afterwards may, if desired, be washed out with a mixture of equal parts of glycerin and water containing $\frac{1}{2}$ per cent. of hydrochloric acid, which will ensure a perfectly nuclear stain. Karyokinetic figures are brought out with great distinctness.

500. Germinal Layers of the Chick (*Wolff's method*[3]).—Harden in 10 per cent. nitric acid; imbed in white of egg.

501. Embryology of Mammalia (*Braun's methods*[4]).—Fix in $\frac{1}{2}$ per cent. chromic acid, harden in alcohol, stain picro-carmine or (better) alum-carmine (Grenacher's formula), imbed in paraffin; cut with Long's modification of the Leyser microtome.

502. Nitric Acid (*Altmann's methods*)—Have been given in paragraph No. 28.

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[1] 'Arch. Anat. u. Phys.,' 1881, p. 123.

[2] Ibid., 1882 (Anat. Abth.), p. 4.

[3] 'Arch. Mik. Anat.,' xxi (1882), p. 64.

[4] 'Arch. Anat. u. Phys.' (Anat. Abth.), 1882, p. 208.

503. Embryology of Lepidoptera (*Bobretzky's methods*[1]).—Ova (of *Pieris cratægi* and *Porthesia chrysorrhœa*) are slightly warmed in water and put for sixteen to twenty hours into 0·5 per cent. chromic acid. The membranes can then be removed, and the ova brought for a few hours into absolute alcohol, stained with carmine, and cut.

504. Araneina (*Balfour's methods*[2]).—Balfour employed a modification of Bobretzky's method, *supra*, 503, which he calls a valuable one. He hardened the embryos in bichromate of potash, after placing them for a short time in nearly boiling water. After removal of the membranes they were stained as a whole with hæmatoxylin, and imbedded for cutting in coagulated albumen.

505. Ova of Amphipoda (Orchestia) (*Uľianin's methods*[3]).—Ova in the earliest stages of development were treated for two hours with picro-sulphuric acid (Kleinenberg's formula). This causes the chorion to swell and burst. Wash out with alcohol, stain with Beale's carmine. Make sections. Ova in later stages, in which the embryo is surrounded by a cuticular membrane, which encloses an albuminous liquid, must have this membrane torn with needles and the albuminous liquid allowed to ooze out before placing in the picrosulphuric acid.

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[1] 'Zeit. wiss. Zool.,' xxxi (1878), p. 198.

[2] 'Quart. Journ. Mic. Sci.,' 1880, p. 167.

[3] 'Zeit. wiss. Zool.,' xxxv (1881), p. 441.

CHAPTER XXXV.

INTEGUMENT AND TACTILE ORGANS OF VERTEBRATA.

506. Epithelium-cells and Gland-cells (*F. E. Schultze's methods*[1]).—In his beautiful researches on the epidermis of fishes and amphibia, and on the epithelium of the intestinal canal of all classes of vertebrates, as well as on that of the respiratory passages of pulmonate vertebrates, Schultze principally used the following reagents:

Pisces. For hardening, solutio Mülleri.

For maceration, iodised serum, Müller's solution.

Amphibia. The same.

Reptilia. The same. Maceration in saliva.

Mammalia. The same. (For hardening, chromic acid of 0·15 per cent. in the case of the stomach of *Delphinus phocaena.*)

507. Ear of Hedgehog (*Schöbl's methods*[2]).—Inject a *very thin* carmine-gelatin mass; cool in ice; and treat the organ for five or ten minutes with osmic acid of 1 per cent. Sections should be made at once, without further preparation. To make surface-preparations, take *young* animals, and after preparing the organ as above, macerate for some time in chromic acid (0·02 to 0·04 per cent.), or in iodised serum. Before examining, treat the preparation with alcohol containing various proportions of acetic acid. Mount in acidulated glycerin or in acetate of potash. Gold chloride is useless; and other stains are of little use.

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[1] 'Arch. Mik. Anat.,' iii (1867), p. 145.

[2] Ibid., viii (1872), p. 296.

508. Crystalline (Hardening of) (*Löwe's methods*[1]).—A fresh bulb is placed in a vessel containing *several litres* of 1 per cent. bichromate of potash solution, which is frequently changed for stronger solutions until the strength of a cold-saturated solution is attained. The bulb must remain in this for at least *a year and a half*, in order that the crystalline may attain the right degree of hardness.

Eye in general.—A freshly extirpated black rabbit's eye may be hardened *for several months* in bichromate of potash, washed in water, stained *in toto* with carmine, imbedded in isinglass-glycerin-jelly, which is to be hardened with large quantities of absolute alcohol, and cut with a microtome.

509. Cutaneous Glands of Frog (*Gage's method*[2]).—To demonstrate the triradiate openings of the glands, macerate a piece of skin from a frog's back or side for two or three days in—

Müller's fluid	1 part.
Water	4 parts.

The epidermis will then separate into layers, and the external layer will show the triradiate openings of the glands. If this layer be then stained with carmine or picro-carmine, the large, flat, nucleated epidermal cells are well demonstrated.

510. Cutaneous Glands of Frog (*Straight's method*[3]).—Wipe a *live* frog dry with a cloth, and put it into water overnight; the external layer of epidermis then comes off very readily.

511. Intra-epidermic Nerve-fibres—Must be studied by the gold-method. Ranvier ('Traité,' p. 900) recommends the boiled-formic-acid and gold-chloride method (Formula No. 113).

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[1] Ibid., xv (1878), p. 557.

[2] 'American Quart. Micro. Journ.,' i, 72. 'Journ. Roy. Mic. Soc.,' i (1878), p. 344.

[3] Ibid.

He also (p. 910) recommends this method for the study of the tactile menisci of the pig's or mole's snout.

512. Cornea.—Impregnation with gold and with silver is indispensable in the study of the cornea.

Negative images of the corneal cells are easily obtained by the dry silver method (Klein). The conjunctival epithelium should be removed by brushing from a living cornea, and the corneal surface well rubbed with a piece of lunar caustic. After half an hour the cornea may be detached and examined in distilled water.

In order to obtain *positive* images of the fixed cells the simplest plan (Ranvier) is to macerate a cornea that has been prepared as above for two or three days in distilled water. There takes place a secondary impregnation, by which the cells are brought out with admirable precision.

The same result may be obtained by cauterising the cornea of a living animal as above, but allowing it to remain on the living animal for two or three days before dissecting it out, or by treating a negatively impregnated cornea with weak salt-solution or weak solution of hydrochloric acid (His).

But the best positive images are those furnished by gold chloride. Ranvier prefers his lemon-juice method (No. 114) to all others for this purpose: lemon-juice five minutes; 1 per cent. solution of chloride of gold and potassium fifteen minutes; reduce in the light in acidulated water. It is important that the cornea should *not remain too long in the gold-solution*, or the nerves alone will be well impregnated.

Ranvier also recommends this method as being the best for the study of the nerves.

Rollett (Stricker's 'Handbuch,' p. 1115) recommends a double impregnation with silver followed by gold for obtaining gold-stained *negative* images. A cornea having been treated *for a short time only* with 0·5 per cent. silver nitrate solution, and the silver reduced, is treated with 0·5 per cent. gold-chloride solution. The brown stain of the silver disappears immediately the preparation is placed in the gold-solution; after a few minutes the preparation is exposed to the light in acidulated water. Reduction of the gold rapidly takes place, and in the place of the former brown stain of the silver the ground-substance shows the well-known blue of reduced gold. The cells are, however, visible, being recognisable by their granular appearance and pale yellow tint.

Rollett (l. c., p. 1102) strongly recommends the following plan:—A fresh cornea is placed (in humor aqueus) in a moist chamber, and exposed to the action of iodine vapour. As soon as it has become brown the epithelium may easily be peeled off. If the reaction is not complete the cornea may be put back into the iodine chamber. When sufficient iodine has been absorbed the preparation may be examined, and it will be found that the network of corneal cells is brought out with an evidence hardly inferior to that of gold preparations. The method never fails, which is not the case with the gold-method. It is admirable as a fixing method.

For dissociation of the fibres Rollett recommends maceration in a solution of permanganate of potash or a mixture of this solution with alum. As soon as the tissue has become brown it is shaken in a test-tube with water, and breaks up into fibres and bundles of fibres.

513. Corneal Corpuscles (*Renaut's method*[1]).—Cornea of frog. Formic acid, 20 per cent., ten minutes; gold chloride, 1 per cent., twenty-four hours; formic acid, 33·3 per cent., twenty-four hours.

514. Pacinian Corpuscles.[2]—Michelson found maceration for several days in concentrated solution of oxalic acid useful for isolating the nuclei of Pacinian corpuscles. The preparation may be subsequently stained with carmine.

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[1] 'Comptes rendus,' 1880 (1r sem.), p. 137.

[2] 'Arch. Mik. Anat.,' v (1869), p. 147.

515. Tactile Corpuscles and Rete Malpighi (*Langerhans's method*[1]).—Pieces of fresh skin are placed for twenty-four hours in a large quantity of ½ per cent. osmic acid, and are then found to be both stained and hardened to the right point for cutting sections.

516. The Corpuscles of Krause (Conjunctiva) (*Longworth's method*[2]).—A fresh bulbus is carefully extracted *in toto* in such a way as to spare as much conjunctiva as possible; the posterior half is cleaned of its fat, muscle, &c., and the conjunctiva drawn back and stretched over it by means of threads passed through different points of its margin. The whole is then thrown into a ⅓ per cent. osmic-acid solution, or hung up in a cylinder and exposed to the vapour of osmic acid. It is best to let it remain twelve or twenty-four hours. The epithelium is then removed by rubbing with a camel's-hair brush or with the finger; and portions of the conjunctiva as large and as thin as possible are removed and examined for corpuscles of Krause either in water or 1 to 2 per cent. acetic acid. They may then be stained and mounted in glycerin if desired. It is advantageous to make a large number of preparations as the corpuscles are not found equally distributed in all eyes nor in all parts of the conjunctiva. If a conjunctiva be divided into five segments two of them will generally be found quite wanting in corpuscles, whilst the other three will contain thirty to sixty of them.

517. Tact-Cells and Tactile Corpuscles (*Merkel's method*[3]).—Small portions of skin (taken by preference from the bill and tongue of ducks or geese) are hardened for one or two days in osmic acid of ½ to 1 per cent., washed in water for the same length of time, placed in strong alcohol for two to three weeks, and sections cut. The tact-cells remain unstained. The tongue of the duck may be cut without previous hardening.

[1] Ibid., ix (1873), p. 730.

[2] Ibid., xi (1875), p. 655.

[3] Ibid., p. 639.

518. Corpuscles of Herbst and Corpuscles of Grandry (*Carrière's methods*[1]).—Take fresh beaks of ducks, remove the skin and papillæ from the margins, and put pieces for twenty-four hours into 1 per cent. osmic acid, wash in water, and put into 90 per cent. alcohol; or put them at once into alcohol (40 per cent. for a few hours, then 70 per cent., then 90 per cent.). The latter are made into sections and stained with neutral carmine, picro-carmine, fuchsin, or hæmatoxylin. The last gives the best results. It is recommended to wash after staining with hæmatoxylin for a short time in *spring water*, as the traces of ammonia contained in it make the stain somewhat redder and bring it out with greater intensity. Or the pieces of skin are treated as follows:

Formic acid (50 per cent.) twenty minutes or until transparency is attained; remove the corneous layer of epithelium; rinse in water; gold chloride 1 per cent. (twenty minutes); rinse in water; Prichard's solution (amyl-alcohol 1 per cent., formic acid 1 per cent., water 98 per cent.) from mid-day till next morning (in the dark); rinse in water; treat with alcohol; imbed in paraffin, and make sections.

It is important to take only *small quantities* of gold chloride, not more than about 10 c.c. of the solution to "quite a number" of pieces of skin and papillæ. On the other hand, *large* quantities of Prichard's solution should be employed.

519. Corpuscles of Golgi (in the tendons of the motores bulbi oculi) (*V. Marchi's methods*[2]).—The enucleated eyes, together with their muscles, were put for not less than three days into 2 per cent. bichromate of potash. The muscles and tendons were then carefully dissected out, stained with gold chloride and osmic acid (Golgi's method) and by the following methods suggested by Manfredi.

520. Ibid. (*Manfredi's method*[3]).—The muscles and tendons removed from the bichromate solution are put for half an hour into solution of arsenic acid or into a 1 per cent. solution of acetic acid. They are then passed directly into 1 per cent. gold chloride, half an hour; distilled water; then reduced in sunlight (until a deep violet colour is obtained) in 1 per cent. arsenic acid solution, which is changed as fast as it becomes brown.

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[1] Ibid., xxi (1882), p. 146.

[2] 'Archivio per le scienze mediche,' vol. v, No. 15.

[3] Ibid.

521. Arsenic and Osmic Acid (*V. Marchi's method* [1]).—The muscles and tendons removed from the bichromate solution as before are treated as follows:—Arsenic acid, 1 per cent., half an hour; osmic acid, 1 per cent., five to six hours.

522. Gold Chloride and Oxalic Acid (*Manfredi's method* [2]).—Fresh tissues treated as follows:—Gold chloride, 1 per cent., half an hour; oxalic acid 0·5 per cent.; warm in a water-bath up to 36°, allow to cool, and examine.

Mount all these preparations in glycerin (balsam clears too greatly). The methods only succeed completely during fine sunny weather.

523. Tactile Corpuscles (*Fischer's method*).—Fischer employed the gold-method of Löwit, *see* No. 112. Ranvier ('Traité,' p. 918) also recommends this method, as well as his two gold-methods, Nos. 113, 114. He finds (as do other authors) that osmic acid and picro-carmine are invaluable aids to the study of these structures and to that of the corpuscles of Pacini.

524. Tactile Hairs (*Odenius' methods* [3]).—Odenius endeavoured to study the terminations of the nerves by means of sections of hair-follicles hardened in weak chromic acid or Müller's solution, and imbedded in gum-water, but without success. Teasing gave no better results. The only method successful with the nervous elements was Max Schultze's method of maceration in oxalic acid or dilute sulphuric acid. The follicles were isolated, and, after a slit had been made through the sheath, were laid in a solution of 3 to 4 grains of "English sulphuric acid" to 1 ounce of water. After some time the shaft of the hair may be removed, and the upper part of the root-sheaths, together with the "conical body," detached by means of a curved needle, from their attachments round the neck of the follicle. It is then generally possible to tease out portions of tissue containing the terminal expansions of the nerves. The duration of the maceration may be stated at from eight to fourteen days. It must not be too prolonged or the nerves will disappear.

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[1] Ibid.

[2] Ibid.

[3] 'Arch. Mik. Anat.,' ii (1866), p. 463.

525. Tactile Hairs.—Ranvier ('Traité,' p. 914) recommends for the study of the nerve-endings the boiled formic-acid and gold-chloride method (Formula No. 113). A tactile hair having been isolated with its bulb, and its capsule incised, is put for about an hour into the formic-acid and gold-chloride mixture, the gold is reduced in slightly acidulated water, hardening is completed in alcohol, and longitudinal and transverse sections are made.

CHAPTER XXXVI.

RETINA OF VERTEBRATA.

526. Chromic Acid for Study of Retina (*Max Schultze's method* [1]).—For the differentiation of the connective-tissue radial-fibres and the nervous radial-fibres, maceration in chromic acid of $\frac{1}{30}$ per cent.

527. Retina (*Max Schultze's methods* [2]).—*Osmic acid.*—Used in somewhat concentrated solutions ($\frac{1}{4}$ to 1 per cent.), it acts first as a fixing and then as a hardening agent.

Strong solutions.—After half an hour's immersion in such a solution small pieces may be teased in a drop of water on a slide. It is then easy to split them into radial laminæ, in which the fibres of the rods and cones can be distinguished (and even isolated if they have not already become too brittle). If, however, portions of retina be allowed to remain for as much as twenty-four hours in the solution they will not suffer; they should be washed out in water, in which they may remain for days.

The staining action of these solutions is specific as regards the outer segments of the rods (at least it is so as regards frogs and fishes; as to mammalia the reaction does not appear to be constant).

(The rods and cones are *perfectly preserved in strong solutions* and the nerve-fibres are not varicose if the solution be strong enough.—A. B. L.)

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[1] 'Arch. Mik. Anat.,' i (1866), p. 179.

[2] Ibid., ii (1866), p. 270.

Weak solutions.—Solutions of ⅕th per cent. or weaker have no longer an exclusively hardening action; they are at the same time macerating agents. Retinæ that have been laid in them for twelve to twenty-four hours are not nearly so brittle as those prepared with strong solutions, and nervous and other fibres may be isolated in them for considerable distances. Nerve-fibres become varicose in these solutions.

Osmic acid may be applied to the retinæ of unopened eyes. If eyes of sheep or calves be placed unopened for a few hours in 1 per cent. solution, they will be found stained and fixed so as to be able to resist the action of water.

Iodised serum.—Very useful for dissecting in and for maceration. The outer segments of the rods and cones are not well preserved in this medium.

528. Retina (Osmic Acid for) (*Max Schultze's methods*[1]).—If a fresh retina (human) be treated for twelve to twenty-four hours with a solution of osmic acid of 2 per cent. or more, the totality of the rods and cones will be found *perfectly preserved.* In weaker solutions both of these structures suffer, the outer segments splitting into disks or disintegrating totally, and the inner segments undergoing the usual post-mortem granular coagulation. But the retinæ treated with the more concentrated solutions become brittle, and the weaker solutions therefore offer greater advantages for the isolation of fibrous elements. If ¼ to ½ per cent. osmic acid be employed, there will always remain a certain number of rods and cones whose inner segments are in a very favorable state for study.

529. Retina of Amphibia (*Laudolt's method*[2]).—Half per cent. osmic acid, *for not more than ten to twenty minutes,* followed by dilute alcohol for two to three days, and teasing in distilled water.

530. Retina (Mammalia) (*Thin's method*[3]).—The isolation, in a good state of preservation, of the ganglion-cells and optic nerve-fibres, which is a matter of great difficulty if a proper method be not employed, is rendered singularly easy by the following process:—Fix in very dilute alcohol. (For the preservation of the processes of the ganglion-cells, mixtures of 1 part of methylated alcohol with 2 of water, and of 1 of methylated alcohol with three of water.) The fibres of the optic nerve expansion are well demonstrated by either of these formulæ; for isolation of them, a mixture of equal parts of alcohol and water is recommended.

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[1] Ibid., vii (1872), p. 244.

[2] Ibid., p. 98.

[3] 'Journ. of Anat. and Physiol.,' xiii (1879), p. 139.

When the strengths of a third and a fourth were used, the bulb was allowed to remain in the fluid for thirty-six or forty-eight hours.

Stain, tease, mount in glycerin or dammar. Of staining fluids, aqueous solution of anilin-blue gave the best results; for the ganglion-cells a double staining with anilin-blue and eosin is useful.

Eyes that have been fixed with alcohol as above directed may be preserved for a long time in glycerin without the nerve-fibres or ganglion-cells suffering in the least. The author obtained excellent preparations from the eye of a kitten that had been sixteen months in glycerin.

(It must not be forgotten that the optic nerve-fibres preserved by this method present the well-known post-mortem varicosities.)

The author thinks that by this process he has been able to demonstrate a sheath to the ganglion-cell processes.

531. Retina (*Ranvier's methods*[1]).—For sections, fix the eye of a triton (without having previously opened the bulb) by exposing it for ten minutes to vapour of osmium. The sclerotic being very thin in this animal such a duration of exposure is generally sufficient. Then divide it by an equatorial incision and put the posterior pole for a few hours into ⅓rd alcohol. Stain for some hours in picro-carmine (1·100).

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[1] 'Traité,' p. 954.

treat again with osmic acid "so as to definitely fix the elements," wash with water, and harden in alcohol. Imbed in wax and oil, and mount in glycerin.

For teased preparations, throw a fresh eye (unopened) into 1 per cent. osmic-acid solution; after twenty-four hours divide it by an equatorial incision and put the halves to macerate in water for two or three days. Tease in water, stain with picro-carmine, mount in glycerin.

532. Retina (*Dennissenko's methods*[1]).—Fresh eyes are taken, and a slit made through sclerotica, choroid, and retina, thrown into a large quantity of Müller's solution where they remain for one or two weeks or longer; they are then washed for a day in water, and brought into alcohol of 60 per cent., followed by strong alcohol. Sections stained with hæmatoxylin followed by eosin.

For teasing, osmic acid of 0·5 to 0·1 per cent.; alcohol of 33·3 per cent.; weak chromic acid; Müller's solution. (For the *details* of the application of these methods to particular ends, *see* the elaborate paper, l. c.)

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[1] 'Arch. Mik. Anat.,' xix (1881), p. 396.

CHAPTER XXXVII.

INNER EAR OF VERTEBRATA.

533. Cochlea (*Waldeyer's methods*[1]).—For teasing fresh preparations, osmic acid of $\frac{1}{10}$ per cent., salt solution of $\frac{1}{4}$ to $\frac{1}{2}$ per cent., or chromic acid of 0·05 per cent. For sections the cochleæ should be opened in two or three places (unless very small), and put for twenty-four hours into a relatively large quantity of palladium chloride of 0·001 per cent., or osmic acid of 0·2 per cent. (small cochleæ) or 0·5 to 1 per cent. (larger ones). They are then treated for twenty-four hours with absolute alcohol, or are brought at once into the decalcifying solution. Waldeyer prefers either 0·001 per cent. palladium chloride with $\frac{1}{10}$th of hydrochloric acid, or chromic acid of from $\frac{1}{4}$ to 1 per cent. Wash with absolute alcohol, and imbed in spinal cord or liver. Waldeyer prefers *not* to employ glycerin-jelly or wax and oil masses for imbedding.

534. Cochlea (*Urban Pritchard's methods*[2]).—Harden in $\frac{1}{3}$ per cent. solution of chromic acid in methylated spirit (ten days). Decalcify in 1 per cent. nitric acid. Imbed in gum hardened by spirit.

535. Cochlea (*Gottstein's methods*[3]).—For fixing, chromic acid of from 1·2000 to 1·3000, osmic acid of from 1·500 to 1·1000. The cochlea to remain in either solution for twenty-four to thirty-six hours, and to be teased in the same liquid. The chromic-acid preparations may be preserved by simply cementing the cover.

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[1] Stricker's 'Handbuch,' &c., p. 958 (1872).

[2] 'Proc. Roy. Soc.,' No. 168. 'Journ. Roy. Mic. Soc.' (1876), xvi, p. 211.

[3] 'Arch. Mik. Anat.,' viii (1872), p. 146.

To get good *surface* views of the entire terminal acoustic apparatus chloride of palladium of 1·1000 is recommended. For sections, harden in chloride of palladium 0·1 per cent., or osmic acid 0·5 to 1 per cent , for twenty-four hours, then in absolute alcohol for twenty-four hours. Decalcify in chromic acid ¼ to 1 per cent., or in chloride of palladium of 0·1 per cent., to which has been added $\frac{1}{10}$th of hydrochloric acid. After decalcification wash for twenty-four hours or more in absolute alcohol, imbed in fresh spinal cord or liver, and harden again in absolute alcohol. If liver be taken, fill the hollow in which the cochlea is to be placed with glycerin-jelly.

536. Cochlea (*Lavdowsky's methods*[1]).—Fresh preparations (if I understand the author rightly) were treated with ½ to 1 per cent. osmic acid, or, which is better, they were treated with 1 per cent. silver solution, and washed for ten minutes in "water to which a few drops of ½ to 1 per cent. osmic acid had been added."

(Dr Lavdowsky appears to be of the school of the gentleman who thought that he correctly indicated the size of some object by stating that it was as big as a bit of chalk.) They were then mounted in "a mixture of glycerin with a few drops of osmic acid" (*sic*), or in the following mixture:—Water 2 parts, glycerin 2 parts, half concentrated acetate of potash 1 part, to which is added 1 drop of osmic acid for each dram of the mixture. The cells must be closed at once. The preparations are not permanent.

For decalcification of the cochlea he recommends that it be first hardened in ½ to 1 per cent. osmic acid, then left for a week in Müller's solution, and finally decalcified in Waldeyer's mixture of chloride of palladium and hydrochloric acid. He imbeds in *pure aqueous* solution of gum-arabic, which he hardens for twenty-four hours in common alcohol. He also imbeds in Rostock transparent soap. The sections are mounted in glycerin.

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[1] Ibid., xiii (1876), p. 497.

It is to be regretted that the author was not more precise and minute in the description of his methods, as the results he obtained by means of them are undoubtedly of the highest value. Besides the paper quoted from the 'Archiv,' *see* the author's treatise in Russian, 'Histology of the Terminal Apparatus of the Cochlear Nerve,' St. Petersburg, 1874.

537. Membranous Labyrinth of Osseous Fishes (*Kuhn's methods*[1]).—The tissues are fixed in the first instance by throwing the head of a fish (after having split the skull along a median line) into chromic acid of $\frac{1}{8}$ per cent., where it remains several hours. The membranous labyrinth is then prepared out *in toto* and placed for from six to twelve hours in osmic acid of from $\frac{1}{8}$ to $\frac{1}{2}$ per cent. The sacculi and ampullæ are then separately imbedded in fresh spinal cord, and thrown into absolute alcohol for hardening, after which sections may be cut (with the free hand). (The recessus utriculi and the sacculi with their otoliths *in situ* must be previously decalcified in chromic or pyroligneous acid.) Sections are stained with hæmatoxylin or picro-carmine.

Kuhn thinks that the high temperature necessary for melting such imbedding-masses as wax, oil, paraffin, &c., is injurious to the more delicate cellular structures.

Chloride of gold was tried but is not recommended. The best macerating agent for preparing teased specimens is *weak* osmic acid ($\frac{1}{8}$th to $\frac{1}{6}$th per cent.), in which the labyrinth must remain not longer than two to three hours. Chromic acid of $\frac{1}{20}$th per cent. gives good results. All preparations should be mounted in concentrated acetate of potash.

538. Labyrinth of Reptiles (*Kuhn's methods*[2]).—Osmic acid

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[1] Ibid., xiv (1877), p. 303.

[2] Ibid., xx (1881), p. 276.

½ per cent. for twenty-four hours. Chromic acid of gradually increased strength (⅛ per cent., ¼ per cent., ½ per cent.) until complete decalcification; wash out in water, put for twenty-four hours into absolute alcohol, imbed in glycerin-gelatin and liver, harden for a few days in absolute alcohol.

538 a. Cochlea (*Max Flesch's method*).—Harden (for twenty-four to thirty-six hours) in the osmic- and chromic-acid mixture (Formula No. 13). To complete the decalcification, if necessary, take chromic acid of 0·25 to 0·5 per cent. Mount in glycerin or balsam. Further staining is not necessary, the osmic acid staining sufficiently. Many details of structure come out with quite diagrammatic clearness, but the cilia of the hair-cells are generally not preserved.

CHAPTER XXXVIII.

NERVE-ENDINGS IN MUSCLE.

539. Muscle, Dissociation of (*Kühne's method*[1]).—From Tergast's paper in 'Arch. Mik. Anat.,' ix (1873), p. 37, I take the following:—

A muscle is buried in a mixture of crystals of chlorate of potash and concentrated nitric acid, and after maceration therein for some time is shaken in a test-tube.

540. Amphioxus (*Langerhans's methods*[2]).—For isolation of the muscle-plates macerate the fresh animal in 20 per cent. nitric acid.

For isolation of the nervous system macerate an animal for three days in 20 per cent. nitric acid, then place it for twenty-four hours in water, and shake forcibly. The *whole* of the nervous system may thus be separated, almost down to the finest peripheral terminations of nerves.

The skin.—Macerate for from half to two hours in osmic acid of $\frac{1}{3}$ per cent. (a strength which for marine animals is equivalent in its effects to that of $\frac{1}{10}$ to $\frac{1}{5}$ per cent. in the case of fresh-water animals). Then place for one or two days in dilute glycerin. The skin may then be stripped off *in toto*, stained with hæmatoxylin, and mounted in glycerin or balsam.

541. Motor Plates (*Fischer's methods*[3]).—In these researches Fischer used for mammals the gold-method proposed by Löwit ('Wien. Sitzgsber.,' Bd. lxxi, Abth. iii, 1875, p. 1), and employed by himself in his researches on the tactile corpuscles ('Arch. Mik. Anat.,' xii, p. 366).

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[1] 'Peripher. Endorg. d. mot. Nerven,' Leipzig, 1862.

[2] 'Arch. Mik. Anat.,' xii (1875), p. 291.

[3] Ibid., xiii (1876), p. 365.

For birds, a muscle (viz. the M. complexus) is cut up into strips 1 to 2 mm. thick and 10 mm. long, which are treated with dilute formic acid (1 part of the acid of sp. gr. of 1·06 to 2 parts water) until they become transparent. (During this maceration the strips are teased to facilitate the penetration of the gold.) They are then passed direct into 1 per cent. gold chloride, and remain there a quarter of an hour. They are then washed with water and placed, according to Löwit's method, in a solution of formic acid 1 part, water 3 parts, where they remain twenty-four hours. They are *not* treated with the concentrated acid.

For reptilia and for pisces the same method was adopted. For amphibia the same method also, except that dilute acetic acid was used in the first instance in the place of formic acid to produce the necessary swelling of the tissues.

542. Nerve-endings in Striated Muscle (*Ranvier's methods*[1]).—Ranvier finds that for the study of the motor terminations of batrachia the best method is his lemon-juice and gold-chloride process (Formula No. 114). The delicate elements of the arborescence of Kühne are better preserved by this method than by the simple method of Löwit.

For the study of the motor plates of reptiles, fishes, birds, and mammals,[2] he finds that his formic-acid and gold-chloride method (No. 113) gives preparations infinitely superior to those obtainable by the method of Löwit, but the lemon-juice method is still better, especially for lizards and mammals. The branches of the terminal arborescence are more regular than in preparations obtained by the formic-acid process.

He finds that the silver-nitrate method of Cohnheim is also useful. He employs it as follows:[1]—Portions of muscle (*gastrocnemius* of frog) having been very carefully teased out in fresh serum are treated for ten to twenty seconds with nitrate of silver solution of 2 to 3 per 1000, and exposed to bright light (direct sunlight is best) in distilled water. As soon as they have become black or brown they are brought into 1 per cent. acetic acid, where they remain until they have swelled up to their normal dimensions (the swelling induced by the acid serving to make up for the shrinkage caused by the nitrate of silver). They are then examined in a mixture of equal parts of glycerin and water.

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[1] 'Traite,' p. 813.

[2] Ibid., p. 826.

This process gives *negative* images, the muscular substance is stained brown, except in the parts where it is protected by the nervous arborescence, which itself remains unstained. The gold-process gives *positive* images, the nervous structures being stained dark violet.

543. Nerve-endings in Muscle (*Wolff's method*[2]).—The pectoral muscle of a frog is excised and stretched over a cork ring cemented to a slide; the cell is then filled with ½ per cent. salt-solution; the preparation may then be studied even with the highest powers.

It is sometimes advantageous to treat a stretched muscle for twenty-four hours with 0·02 per cent. osmic acid; in this case it must be stretched with hedgehog spines for pins. To prevent the preparation from overstaining subsequently, it may be placed for some time in Beale's carmine.

Chloride of gold and potassium were used of the strength of 0·03 per cent. for twenty-four hours.

Carl Sachs' method for demonstrating sensitive nerve-endings was also employed. The living muscle is put for twenty-four hours into 1 per cent. acetic acid, washed, put for twenty-four hours into very dilute picric acid and studied in dilute glycerin. The preparations so obtained are extremely transparent.

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[1] Ibid., p. 810.

[2] 'Arch. Mik. Anat.,' xix (1881), p. 355.

544. Nerve-and-Muscle (*Bremer's method*[1]).—Small pieces of fresh muscle of frog or lizard were treated as follows:—Formic acid 25 per cent., until transparent; gold chloride, 1 per cent., fifteen to twenty minutes; formic acid 25 per cent., twenty-four hours in the dark; formic acid 50 per cent., twenty-four hours in the dark; "20 per cent. formic-acid glycerin" two to three weeks, until sufficiently lightened in colour. The connective tissue is by this time so far macerated that the muscle-fibres easily fall apart.

Mount in acidulated glycerin (1 per cent. formic acid).

Muscles of hydrophilus may remain three hours in ½ per cent. gold-chloride solution.

The same method was used for the study of the nerves of small arteries and veins ('Arch. Mik. Anat.,' xxi (1882), p. 671).

545. Smooth Muscle, Isolation of Fibres (*Schwalbe's method*[2]).—Maceration in weak chromic-acid solution. (0·02 per cent. proved a generally useful strength.) This is a better reagent than osmic acid, 1 per cent. acetic acid (Moleschott), weak sulphuric acid, pyroligneous acid (Meissner), 20 per cent. nitric acid (Reichert), 32 to 35 per cent. potash solution (Moleschott), as it preserves better than any of these the finer structure of the cells.

546. Bladder of Frog (*Tolotschinoff's methods*[3]).—Inflate the bladder of a living frog (young specimens of *R. temporaria* are the best) by blowing air into it by means of a curved glass tube passed into the cloaca. Pencil its surface with ½ per cent. gold chloride until the muscles begin to appear white (ten minutes generally suffice). Excise the bladder and bring it into ½ per cent. gold chloride; after ten minutes remove it into acidulated water, and leave it there for three days. It is advantageous to stain the impregnated tissues with carmine.

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[1] Ibid., xxi (1882), p. 195.

[2] Ibid., iv (1868), p. 394.

[3] Ibid., v (1869), p. 509.

547. Smooth Muscle (Bladder of Salamandra) (*Flemming's method*[1]).—A bladder is hardened in bichromate of potash of 1 per cent. or more, the epithelium is removed by pencilling, the tissues are stained with hæmatoxylin, eosin, or anilin, and examined in water (which is preferable to glycerin).

548. Musculus Dilatator Pupillæ (*Dogiel's methods*[2]).—The difficulty in the demonstration of this muscle lies in the getting rid of the pigment of the iris. V. Wittich employed chlorine to decolourise the pigment. Merkel macerated for some days in oxalic acid and removed the pigment with a camel's-hair brush. Other observers resorted to eyes naturally free from pigment,—white rabbit, white mouse, blue human eyes. For isolation of the muscle-fibres there have been employed: acetic acid (2 to 5 per cent.), nitric acid (20 per cent.), caustic potash (32 per cent.), iodised serum, and chromic acid (0·01 to 0·05 per cent.).

Dogiel prefers the following method:—An excised iris is placed for twelve hours in strong acetic acid or for several days in a weak solution. It is removed, brushed with a soft brush, and carefully *split* with the point of a scalpel. By this means it is possible to remove from the anterior surface the connective tissue and layer of blood-vessels; from the posterior surface some part of the connective tissue, vessels, and pigment that obscure the course of the muscle in the iris. The remaining layer of the iris so prepared is now stained with carmine and mounted in acidulated and diluted glycerin.

The following reagents are also useful for making the iris denser and more easy to split:—Chromic acid (0·01 per cent.), gold chloride (0·1 per cent.), palladium chloride.

Dogiel also stains the smooth muscle-fibres by putting the iris for some hours into strong acetic acid and then staining with an acid mixture of carmine and glycerin.

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[1] 'Zeit. wiss. Zool.,' xxx, Supp., 468.

[2] 'Arch. Mik. Anat.,' vi (1870), p. 91.

549. Smooth-muscle, Nerve-endings in (*Gscheidlen's methods* [1]).—The following directions are given for the employment of gold after Löwit's manner:—Place the fresh tissue (after having rinsed the surface with distilled water) in formic acid (of from 2 to 4 per cent.) for twenty-four hours. Thence remove it direct to 1 per cent. gold chloride, where it remains till it becomes straw colour (which should be in about fifteen minutes), rinse with water, and bring it back into the formic-acid solution. Leave it to reduce therein *in the dark* for twenty-four hours, wash, and mount in glycerin. Gscheidlen thinks these preparations are permanent; *none* of his have faded, but all are as fine as when first mounted more than two years before.

550. Nerve-endings in Smooth Muscle.[2]—Ranvier recommends one or the other of his two gold-processes (Nos. 113 and 114). The bladder of frogs should be carefully distended by injection of the lemon-juice or gold chloride and formic acid through the cloaca.

551. Innervation of Bladder of Frog (*Wolff's methods* [3]).—A frog is killed and a solution of gold chloride of 1·20,000 injected into the bladder through the anus. (If the injection flows out on removal of the syringe, tie the frog's thighs together.) Now open the frog, dissect away the attachments of the bladder, ligature the intestine above the bladder, and cut away the abdomen of the frog so as to have in one piece bladder, rectum, and hind-legs. (All this time the bladder must be kept moist with weak gold-solution.) The bladder and the rest are now put into gold-solution of 1·2000 for four hours; the bladder is then excised, slit open, and pinned (with hedgehog spines) on to a cork (outside downwards). Place it under running water until all the epithelium is washed away. Use a pencil if necessary. Put for twenty-four hours into gold-solution of 1·6000. Wash in pure water, and put away in the dark "for some time" in acidulated water, and finally reduce in fresh water in common daylight. The muscles should be pale blue-red; medullated nerves dark blue-red; sympathetic nerves and ganglia carmine-red.

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[1] Ibid., xiv (1877), p. 325.

[2] 'Traité,' p. 854.

[3] 'Arch. Mik. Anat.,' xx (1881), p. 362.

CHAPTER XXXIX.

MEDULLATED NERVE.

552. Medullated Nerve-fibre (Indentations and Constrictions) (*Lanterman's methods* [1]).—The indentations as well as the constrictions may be well studied in the living fibre. The best preparations for the study of the indentations are made by placing a fresh nerve for fifteen to thirty minutes in 1·1000 osmic acid, or for one or two hours in a weaker solution. Tease and mount in acetate of potash, glycerin, or dammar; the last gives the best results. Fresh nerves may also be treated with chloroform or collodion, or may be stained in turpentine coloured with alkannin. Chromic acid of 1·5000 may be used. The constrictions should also be studied in silver preparations.

553. Medullated Nerve-fibre (*Kuhnt's methods* [2]).—For the sheath of Schwann the following methods, taken from various observers:

1. Concentrated acetic acid, followed by water.
2. Ether, chloroform, turpentine.
3. Caustic soda.
4. Caustic soda, followed by fuming nitric acid and potash.
5. Boil in absolute alcohol and ether, and when cool add caustic soda, or boil again with glacial acetic acid.
6. Fuming nitric acid followed by caustic potash.

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[1] 'Arch. Mik. Anat.,' xiii (1876), p. 8.

[2] Ibid., p. 441.

7. Chlorum dilutum.
8. Aqua Javelli.
9. Nitric acid of 36 per cent.
10. The following is particularly recommended:—Place nerves for twenty-four hours in ¼ per cent. osmic acid, wash with water, and place for twenty-four hours in water 10·0 grammes, liquor ammoniæ 10 to 30 grammes. Tease in water. Portions of sheath may by this means be obtained emptied of their contents across an entire field of the microscope.

For the indentations of the medulla:

1. Concentrated corrosive sublimate.
2. Chromic acid.
3. Bichromate of potash.
4. Nitrate of silver.

For the sheath of the axis-cylinder:

Maceration in 36 per cent. nitric acid, alcohol dilutus (Ranvier's), weak osmic- and chromic-acid solutions. The chromic acid should be from 1·3000 to 1·6000 strength; the alcohol dilutus should be allowed to act for several weeks. Subsequent staining with anilin-red is useful. The 36 per cent. nitric acid should be allowed to act for twenty-four to fifty-four hours.

Another good method is to macerate for from six to twenty hours in an osmic-acid solution of from 1·350 to 1·700, and tease.

554. Medullated Nerve (*Ranvier's methods*[1]).—A nerve, having been exposed by dissection on a living or recently killed animal, is treated first with distilled water and then with silver nitrate of 3·1000. As soon as it is seen to be fixed by the action of the silver, it is dissected out and brought into the silver solution, in which it is exposed to the light for five, ten, fifteen, or twenty minutes. It is then washed and examined in distilled water. This treatment serves to demonstrate the endothelium and the Latin crosses on the annular constrictions, as well as in some cases the appearance of transverse striation (Frommann) of the axis-cylinder. Preserve in glycerin.

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[1] 'Traité,' p. 725.

For the demonstration of the biconical thickenings of the axis cylinder, tease fresh nerves in the silver solution and examine in water or glycerin (p. 727).

For fixing nerves with osmic acid Ranvier proceeds as follows: A small slip of wood (lucifer match) has a groove cut in it longitudinally to receive the nerve, the nerve is arranged in the groove and fixed by a ligature at each end of the wood. It is then detached, and the whole is thrown into osmic-acid solution (1 per cent.). Ranvier recommends this method for demonstrating the cylindro-conical segments. Golgi points out that by this *stretching* the sleeves and funnels are distorted and obscured (*see post*, No. 556).

555. Medullated Nerve (the chloroform method) (*Tizzoni's methods* [1]).—Fresh nerve is slightly teased and hardened for some days in alcohol of gradually increased concentration, beginning with about 36° and ending with absolute. (It is sometimes well to vary the process by first fixing the tissues by treatment for a few days with 2 per cent. bichromate of ammonia or weak bichromate of potash.) The nerves are passed from the absolute alcohol into a test-tube containing a large quantity of chloroform. (Chloroform is preferred to ether and to benzin because its action is quicker and because it is difficult to procure pure benzin). They are boiled for from one to two hours in the chloroform, which must be renewed two or three times, and are from time to time examined with the microscope to ascertain whether the myelin is entirely dissolved. As soon as the dissolution is seen to be complete, the pieces of nerve are put back into alcohol, where they remain until they are wanted for staining. Stain with hæmatoxylin or picro-carmine. Hæmatoxylin stains deeply both the axis-cylinder and the nuclei of the fibre; picro-carmine stains the nuclei deeply, the axis cylinder hardly at all, and the "horny reticulum" is tinged with yellow. Mount in dilute glycerin.

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[1] 'Archivio per le scienze mediche,' vol. iii, No. 1 (1878), p. 4.

Second method (*Ranvier's method*).—Osmic acid 1 per cent. for one or two hours, wash, stain picro-carmine twelve hours, tease in weak glycerin or weak solution of acetate of potash.

Third method.—Osmic acid as before, followed by Beale's carmine for two or three days (either pure or with the addition of a few drops of picro-carmine). Tease in glycerin.

556. Peripheral Medullated Nerve. Osmium Bichromate of Potash and Silver Nitrate (*Golgi's method*) [1].—A perfectly fresh piece of nerve is thrown into the following liquid:

2 per cent. solution of bichromate of potash .	10 parts.
1 per cent. solution of osmic acid	2 parts.

After about an hour's immersion, the piece of nerve may be cut into lengths of ½ to 1 cm., which are put back into the liquid.

Four hours after the first immersion of the nerve in the mixture, begin to put the pieces into nitrate of silver solution, transferring a certain number of pieces every three hours, so as to be sure that some of them shall have had a bichromate bath of a proper duration. (This duration may, roughly speaking, be said to lie between six and twenty-four hours.)

The strength of the nitrate of silver solution is 0·50 per. cent. The duration of the silver-bath must not be less than eight hours; it may be indefinitely protracted.

Dehydrate, clear with turpentine, mount in dammar.

The method is more expeditious and easier of application than the bichromate and silver-nitrate method (No. 565), and the results are somewhat more precise, but the preparations do not keep in dammar.

These two methods serve for the demonstration in *peri-*

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[1] Ibid., iv, No. 10 (1879), p. 237.

pheral medullated nerve-fibres of the funnel-shaped coils of sustaining filaments discovered by Rezzonico in the medullated fibres of the spinal cord.

In all methods for the demonstration of the funnels, it is important to observe the utmost delicacy of manipulation, and in particular, the fibres *must not be stretched;* their stretching is a weak point in the methods of Ranvier, No. 554.

557. Medullated Nerve (*Rawitz's methods*[1]).—Treat a piece of nerve for twenty-four hours with a dilute alcoholic solution of fuchsin (six to seven drops of a 4 per cent. solution to a watch-glassful of water), wash out, and tease in a 50 per cent. solution of acetate of potash. The preparations are very beautiful, but the author knows no means of preserving them, for 50 per cent. acetate of potash, glycerin, and levulose and Canada balsam all after a time wash out the stain and cause the contents of the fibres to exude.

Nitrate of silver $\frac{1}{5}$ per cent. Like the former method, this demonstrates the constrictions, the annulus of Ranvier, the axis-cylinder, the double contour, and the nuclei; and, if nerves be stained *in toto*, the endothelium of the neurilemma.

Teasing in 0·75 per cent. salt-solution will demonstrate the constrictions, the double contour, the sheath of Schwann, and the indentations of Lantermann.

Humor aqueus has an analogous action.

It is recommended to tease in 1 per cent. osmic acid, and, having removed the acid with blotting-paper, add very dilute fuchsin solution (Key and Retzius) (for the study of the sheath of Schwann and other details).

557 a. Peripheral Nerves. Bichromate of Potash and Silver-Nitrate Process (*Golgi's method*[2]).—With some modifications the process abstracted *post* No. 565 is applicable to the study of peripheral nerves.

1. Pieces of nerve are immersed in the bichromate solution

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[1] 'Arch. Anat. u. Phys.,' 1879, p. 62.

[2] 'Archivio per scienze mediche,' iv, No. 10, p. 238 (1879).

for from four, six, or eight hours to one day, or at most two days.

2. From time to time pieces are removed into the nitrate of silver; they remain there for from twelve to twenty-four hours.
3. They are washed with several changes of alcohol.
4. Tease in the alcohol, dehydrate, clear with turpentine, mount in dammar.
5. Reduce in direct sunlight; in summer a few days suffice, in cold weather some weeks are necessary.

Does not give quite such fine results as the osmium bichromate silver method (*ante,* formula No. 556), but the preparations keep indefinitely.

558. Medullated Nerve-fibres (Structure of Medulla) (*Ceci's method*[1]).—Ceci finds that the structures described by Rezzonico and Golgi can be observed by means of much simpler methods.

Fresh nerve-fibres are carefully mounted in aqueous humour under a cover luted with paraffin.

The gold-chloride and formic-acid method will also serve to demonstrate the sleeves.

As a general rule, it is very important to employ fixing agents (osmic acid, 0·5 to 1 per cent., gold chloride, silver nitrate) for the study of the sleeves. In macerated or in alcohol preparations it is seldom possible to observe more than a "reticulum corneum," which is an artefact produced by distortions of the sleeve-membranes.

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[1] 'Atti R. Accad. d. Lincei.' vol. ix, ser. 3 (1881), p. 89.

CHAPTER XL.

MYELON.

559. Central Nervous System (*Betz's methods*[1]).

HARDENING.

The *spinal cord*, *medulla oblongata*, and *pons Varolii* are treated as follows :—The dura mater is removed, and they are hung up in a cylinder containing 75 to 80 per cent. alcohol, to which is added enough iodine to produce a light-brown colouration. After from one to three days the preparation will be found to be somewhat surface-hardened; it is taken down and the pia mater and arachnoid are removed. If the pia mater does not come away completely enough the preparation is put back for some days into the alcoholic iodine. The membranes having been removed, the preparation is put back into the original fluid, which is found to have become colourless owing to absorption of the iodine by the tissues. Fresh quantities of a strong solution of iodine in alcohol are from time to time added to the liquid in order to keep it at its original strength of iodine (as shown by the colour). If the membranes have been carefully removed it will be found that after about six days the preparation ceases to take up further quantities of iodine. The preliminary hardening may now be considered complete.

The preparation is now brought into a 3 per cent. solution of bichromate of potash. (A small weight is attached to it to prevent any portion of it from floating above the surface of the liquid. After a day or two it will have lost much of its alcohol, and will sink to the bottom of the vessel, which is equally undesirable; this must be watched for, and the preparation hung up or otherwise supported.) The vessel is put away *in a cool place*. As soon as a brown turbidity is seen in the liquid, together with a brown deposit on the preparation, the hardening may be considered to be complete. The preparation must be at once washed with water, and put away until wanted in a $\frac{1}{2}$ to 1 per cent. solution of bichromate.

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[1] 'Arch. Mik. Anat.,' ix (1873), p. 101 ff.

Cerebellum.—Must be quite fresh, and before placing in the iodine the membranes and vessels must as far as possible be very carefully removed. (If the pia mater does not come away freely, the organ must be macerated for a few hours in iodine solution in which other preparations have been kept, and which is diluted before using for this purpose.) The membranes having been removed, the cerebellum is placed (supported on cotton-wool, with which the different organs are so propped up as to preserve their natural position) in solution of iodine for two or three days, and fresh iodine solution frequently added.

The pia mater is now removed from the rest of the preparation, which is put back for seven to fourteen days into the iodine solution. If at the expiration of this time it be found that the cerebellum can be supported on the finger by the vermiculus alone without bending, the preliminary hardening is complete, and it is brought into a 5 per cent. solution of bichromate, where it remains until fit for cutting.

Cerebrum.—The cerebrum is divided into two halves along the median line of the corpus callosum, and put into the iodine solution. After a few hours the pia mater is removed from the fissure of Sylvius and from the corpus callosum, and, if possible, the choroid plexus is removed likewise.

The preparation is now put away in the iodine solution in a cool place (in summer in a cool cellar), and fresh iodine added as soon as the liquid is seen to lose colour (which must be watched for). After twenty-four to forty-eight hours the remaining pia mater is carefully removed by means of scissors and forceps from the fissures and convolutions, and one half-volume of fresh iodine solution is added to the liquid. (To facilitate the penetration of the liquid, wads of cotton-wool are stuffed into the fissure of Sylvius, between the operculum and the median (central) lobe, in the direction of the descending cornu, and between the convolutions.) After twenty-four to seventy-two hours the brain is brought into fresh solution of iodine in 70 per cent. alcohol, where it remains until the hemispheres are hard enough to be supported on two fingers without bending. (This will not be before ten to fourteen days.) It is then put into 4 per cent. solution of bichromate and left to acquire its definitive hardness. If an excessive brown deposit make its appearance, and the brain be found notwithstanding to be not hard enough for cutting, it must be rinsed with water and the bichromate solution changed. When ripe for cutting the brain ought to show an almost equal intensity of yellow-brown stain over the whole surface of a cut made through the total thickness of a hemisphere.

Brains that are not fresh require for hardening longer time and stronger alcohol.

Instead of the iodine solution, it is possible to use for the preliminary hardening a mixture of equal volumes of chloroform and ether; but this mixture is not to be recommended, on account of its solvent action on protoplasm and on the processes of ganglion-cells.

Cutting, Staining, and Mounting.

The bichromate is removed by washing for from one to several days in water, the preparations are placed for a short time in alcohol, imbedded in a mixture of olive oil and *yellow* wax, and cut in a microtome. The sections are washed in water (in which is placed a piece of camphor to prevent the development of Infusoria), for twenty-four to seventy-two hours, and stained with the author's carmine solution, Formula No. 55. They are dehydrated in baths of alcohol of gradually increasing strength, cleared in "a somewhat resinous" turpentine, and mounted in solution of dammar in turpentine.

560. Spinal Cord (*Krause's methods*[1]).—After having, in the course of his researches on the ventriculus terminalis, tried all the known methods of preparation, Krause recommends the following as being the safest:

The fresh cord is hung up in a large cylinder full of Müller's liquid; the lower end has a small weight attached to it, to avoid torsions which are otherwise caused by the elastic fibres of the membranes. After twenty-four hours, the Müller's liquid is changed for 1 per cent. chromic acid, which is changed for fresh on the fourth day. A few days later (when the cord appears hard), the chromic acid is removed by means of water, and the cord put into spirit, followed by absolute alcohol, which must be changed at least twice. Imbed in paraffin; the sections either pass through carmine, alcohol, oil of cloves, into Canada balsam, or simply through benzol, or Bronner's "Fleckwasser," and Canada balsam. ("Fleckwasser" appears to be a preparation of xylol.)

561. Nervous Centres (*Hamilton's method*[2]).—The author objects to the use of chromic acid for hardening, on account of the uncertainty of its action, some parts hardening well, others becoming brittle, discoloured, and totally useless for purposes of research; whilst it cannot be employed for large masses of tissue without hardening one part more than another. He recommends the following procedure:

Take a fresh brain, and make a series of incisions into

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[1] Ibid., xi (1875), p. 226.

[2] 'Journ. of Anat. and Physiol.,' xii (1878), p. 254.

different parts, still keeping everything *in sitû;* or slice it into any number of segments about one inch thick, but of the whole length or breadth of the organ, as may be desired. Do *not* remove the membranes; they form a protection for the superficial layers, and do not interfere with the hardening process. The large segments are placed flat in a large vessel padded with cotton; do not put them one above the other. Cover them with the following fluid:

Müller's fluid	3 parts.
Methylated spirit	1 part.

(Heat is evolved on mixing these liquids, and the mixture must be allowed to cool before pouring it over the brain tissue.) Put the preparations away in an ice-safe. Turn the segments over next day. Change the solution in a fortnight or three weeks; or if on examining a section of one of the pieces it is found that the hardening reagent has penetrated to the interior, they may be at once removed to the following mixture:

Bichromate of ammonia	1 grm.
Water	400 c.c.

in which they remain for one week. Then change the solution to one of 1 per cent. for one week; and let this be followed by a solution of 2 per cent. for another week, or longer if required. The pieces will now be sufficiently hard for cutting; they may be kept permanently in solution of chloral hydrate, twelve grains to the ounce.

It will be found that the consistence of the preparations is tough and firm, but not hard and brittle as when prepared in chromic acid. There is no shrinking of the tissues from an undue amount of hardening, nor are they discoloured as chromic-acid preparations usually are. (The object of using Müller's fluid is that it hardens brain substance very gradually, whilst it has a high degree of penetration. The small amount of spirit prevents decomposition without producing contraction of the tissue.)

Hamilton cuts his sections in a Rutherford freezing microtome. It is important to properly prepare the tissues for freezing, otherwise it will be found that the crystals of ice so break up the delicate nervous tissue as to render it totally useless for minute examination. This is accomplished by imbedding in *syrup*. The sugar somewhat retards the freezing, and besides seems to alter the manner of crystallisation, so that instead of the ice being spicular in form it becomes granular and does no injury to the parts. The syrup requires to be of a particular strength.

The hardened tissues are to be steeped in water for a night or longer, to remove any trace of the hardening reagent, that is to say, until the water ceases to be stained by it. Then put for forty-eight hours into the following syrup:

Double refined sugar .	2 ounces.
Water	1 fluid ounce.

Wash the superfluous syrup from the surface of the preparation and put into the ordinary imbedding mucilage for an hour or so before cutting. Imbed in the freezing microtome with mucilage in the usual way. Float the sections into water.

Staining.—Stain first with a "strong solution of perosmic acid," and then slightly with carmine.

Clearing.—Hamilton's ideal of clearing for brain-sections is to preserve permanently that moment of the process in which all the parts are *half cleared* (*see* **Xylol**, No. 567). He appears to use either oil of cloves or turpentine, and to mount in balsam or dammar.

562. Encephalon (*M. Duval's methods*[1]).—*Hardening* (*first method*).—Place the fresh tissues in Müller's solution (bichromate of potash 25, water 1000), change the liquid after the first twenty-four hours, and again after three or four days. After two or three weeks place the preparations in chromic acid of 3 per 1000, change the liquid every day for the first week, and after that every eight days until the middle of the second month, after which time it is no longer needful to change the liquid. The preparations must remain at least two months in the chromic acid; the longer they remain in it the better. A few fragments of camphor should be added to the liquid in order to prevent the growth of mould.

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[1] Robin's 'Journal de l'Anatomie,' 1876, p. 497.

Hardening (second method, glycerin and acetic acid[1]*).*—Place the fresh tissues in a mixture of equal parts of glycerin and acetic acid; after twenty-four hours remove them to Müller's solution, and after forty-eight hours more to chromic acid. (The strength of the solution is not indicated.) Change the chromic acid once or twice, and the preparations will be fit for cutting in about eight or ten days. (Small encephala (rat, bat) need not be extracted from the cranium provided this be largely opened before immersing them in the glycerin, nor need they be extracted before cutting, as the cranium will be found to be completely decalcified.)

This method is highly expeditious, and furnishes hardened tissues of a singularly homogeneous consistence, quite without fragility, but it should only be employed for the purpose of obtaining general views of structural relations, as the anatomical elements are somewhat changed by it: cells and axis-cylinders swell.

Imbedding.—Before imbedding the tissues are to be soaked for six or eight days in alcohol of 36°. They are then soaked for a few hours in water, and then in a mixture of equal volumes of a very thick solution of gum and glycerin. A piece of pith is then cut into a long strip about as thick as stout paper by "peeling" it continuously with a razor, as one peels an apple: the strip of pith has a streak of the gum-glycerin laid along it; the piece of tissue (which need not have been for more than a few minutes in the gum) is rolled up in the strip, and a piece of thread wound round the whole, which is then thrown into common alcohol, where it remains for several hours. The gum being now solidified, the preparation is placed in the well of the microtome and wedged there by means of small sticks of dry pith well pressed between the fingers to reduce their volume, and the well is finally filled with alcohol.

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[1] l. c., p. 498.

Duval considers that this method of imbedding gives far greater fixity than the usual methods, and notably far greater than the paraffin process.

Cut with a razor wetted with alcohol. Stain carmine or carmine and anilin (No. 572), fix the stain with acetic acid, dehydrate, clear with turpentine, and mount in balsam or dammar.

563. Giacomini's Brain-processes (*Giacomini's methods*[1]).—Although these are in intention *macro*scopic methods, it appears worth while, both on account of their thorough success and on account of their suggestiveness, to give an account of them here.

The object is to make "dry" preparations of the encephalon; by which is meant preparations that can be permanently preserved *in the air*. The methods hitherto employed were not successful because they consisted in making preparations that were "dry" in the literal sense of the word, that is, deprived of their natural water; and since brain-substance contains 88 per cent. of water, such preparations could not of course be obtained without so great an amount of shrinkage as to most seriously diminish the scientific value of the result. The principle of Giacomini's method is on the contrary to *retain* the natural water of the tissues, or an equivalent for it, by means of impregnation with a hygroscopic substance,—glycerin.

The process consists of two divisions: 1, hardening; 2, impregnation with glycerin.

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[1] Communicated to the R. Academy of Medicine of Turin, 7th June, 1878: 'Archivio per le scienze mediche,' iii, No. 2 (1878), p. 11.

1. For hardening may be used, zinc chloride, bichromate of potash, chromic acid, nitric acid, or alcohol.

Chloride of zinc gives the best results. Perfectly fresh brain is put into a saturated aqueous solution of the salt (if there be reason to fear that the tissues are somewhat softened through having been left too long after the death of the subject, it is well first to inject 600 grammes of the solution through the internal carotid arteries). After forty-eight hours' immersion (during which time the floating brain must be turned over three or four times, so that all parts of it may duly come into contact with the liquid) the surface of the brain will have attained a consistency that will allow of the removal of the arachnoid and pia mater. The meninges having been removed, the encephalon is put back into the solution for two or three days more, during which time it will be seen that, increasing in specific gravity, it tends towards the bottom of the vessel containing it. When this is seen to happen, it must be removed into commercial alcohol, as if allowed to remain longer in the chloride of zinc solution it would take up too much water.

In the alcohol it may remain for an indefinite time, or it may be removed if desired after ten or twelve days. (During the alcohol-bath, it must be frequently turned over in order that no malformations may arise from continuance of pressure on the same part.)

It is then removed into glycerin (either pure or with 1 per cent. of carbolic acid). It floats at first, but gradually sinks as the alcohol evaporates. As soon as it has sunk just below the surface it may be removed and exposed to the air.

It is set aside to "evaporate" in a convenient place for a few days. As soon as the surface has become dry, it is varnished with india rubber or (better) with marine glue varnish diluted with a little alcohol. This completes the process.

If it be desired to make dissected preparations, the necessary dissections should be made on removing the encephalon from the alcohol before putting into glycerin.

Bichromate of potash may be used for hardening in solutions gradually increasing in concentration from 2 to 4 per cent. The liquid must be frequently changed, the immersion must be of not less than a month's duration. Six to eight days will suffice for the alcohol-bath, or this may be altogether omitted.

Nitric acid is used in solutions of from 10 to 12 per cent. for twelve to fifteen days. (Encephala float in this liquid and must therefore be frequently turned over. It is this reagent that gives the *toughest* preparations.)

Concerning the value of the process, Golgi (from whose abstract I take the foregoing account) states that after a series of experiments he is able to affirm that for preservation of the volume, the colour, the finer relations of the parts, and the physiognomy proper to the organ, the process is far superior to any hitherto known. I am able to add that I saw specimens of Giacomini's preparations at the Milan International Exhibition of 1881, and think it would be hard to overpraise their beauty of aspect.

564. Encephalon (*Deecke's method*[1]).—"To harden the entire brain so that the inside and the outside shall be hardened equally and properly, Dr Deecke finally adopted bichromate of ammonia in ½ to 1 per cent. solution, according to the consistence of the brain. When nominally soft he adds say ⅙th to ⅒th per cent. of chromic acid to the solution, and always ⅙th to ¼th of the whole volume of alcohol. It is then placed in a refrigerator and the fluid changed frequently. After a month add a little more alcohol from week to week until the alcohol is 90 per cent. This is changed as often as it is discoloured. The treatment requires from twelve to eighteen months."

"The brain to be cut is placed upon the piston (of the microtome—a Ranvier model) and held *in sitû* by several pieces of soft cork. It is then imbedded in a cast of paraffin, olive oil, and tallow, which after it has become hard, is held in position by a number of small curved rods attached to, and projecting upwards from, the piston to the height of about an inch. Before cutting, and as it proceeds, the cast is carefully removed from around the specimen to the depth of about half an inch (which is easily done by the use of a good-sized carpenter's chisel), so that the knife never comes in contact with the cast."

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[1] 'Journ. Roy. Mic. Soc.' (N.S.), iii (1883), p. 449.

Cutting is done under alcohol, the entire microtome being immersed in a copper basin. The sections are floated, with the aid of a fine camel's-hair brush, on to sheets of glazed writing paper. They are removed thereon successively into staining, washing, and clearing fluids. After clearing, they are brought on the paper on to a slide, and the paper is gently pulled away from them; they are then mounted in chloroform- or benzol-balsam.

It should be noted that the membranes should *not* be removed from the brain; they present no obstacle to cutting if this is done with a slight sawing movement, or with a series of short cuts, instead of one sweep of the knife. By this plan the sections are much more perfect and uniform in thickness, and the loss in a series of from four to five hundred to the inch through the entire cerebrum of man may not amount to more than 2 or 3 per cent.

565. Spinal Cord. Bichromate and Silver-nitrate Process (*Golgi's method*[1]).—I take the following *résumé* of the method from the interesting paper of Golgi's pupil, Rezzonico, l. c., "Sulla struttura delle fibre nervose del midollo spinale."

1. Take pieces of perfectly fresh spinal cord, and soak them in a 2 per cent. solution of bichromate of potash, for a period of time varying according to temperature. (In

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[1] 'Archivio per le scienze mediche,' iv, No. 4 (1879), p. 85.

summer eight to fifteen days may suffice, in winter about a month is necessary.)

2. Wash them, and put them into a 0·75 per cent. solution of nitrate of silver. The period of immersion therein depends on the temperature; in summer, the reaction will be complete throughout the tissues in two or three days; in winter, eight, ten, or more days.

3. Dehydrate small pieces with alcohol (make sections if necessary), clear in oil of turpentine, tease in the turpentine, and mount in dammar.

4. The preparations are then left to themselves in order that the secondary impregnation may take place. In direct sunlight, eight to ten days will complete the process; in diffused daylight (or in the dark?), twenty, thirty, or forty days.

A somewhat greater precision of the reaction is obtained by treating the fresh tissues with osmic acid (by means of interstitial injection) before putting them into the bichromate. In this case, a much shorter immersion in the bichromate will suffice (four, six, or eight days).

By this means may be demonstrated in the medullated fibres of the spinal cord, a chain of conical funnels, set one within another, and embracing the axis cylinder with their narrow aperture, and the external surface of the following funnel with their greater aperture; and it is seen that they consist of a fine spiral fibre wound into the form of a funnel. (The appearance of rings and sleeves (*manichetti*) is a result of imperfect reaction of the silver.)

566. Cortex of Cerebrum (Bichloride of mercury staining-method) (*Golgi's method*[1]).—This method, which may be said to be in principle identical with the bichromate of potash and silver nitrate method of the author, consists like the latter of two processes: 1, hardening in bichromate; 2, treatment with bichloride of mercury.

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[1] Ibid., iii, No. 11 (1878), p. 3.

For hardening, use either a solution progressively raised in concentration from 1 per cent. to 2½ per cent., or Müller's solution. Take small pieces of tissue (not more than 1 to 2 c.c.), large quantities of liquid, and change the latter frequently so as to have it always clear. Fifteen to twenty days' immersion will suffice, but twenty to thirty should be preferred.

The tissues are then passed direct from the bichromate into the bichloride of mercury. The solutions of the latter employed by Golgi varied from 0·25 per cent. to 0·50 per cent.; he cannot say which strength is to be preferred. The immersion in the bichloride must be much longer than the immersion in the nitrate of silver bath of that process; for the latter, twenty-four to forty-eight hours suffice; but in the bichloride, an immersion of eight to ten days is necessary in order to obtain a complete reaction through the whole thickness of the tissues. During the bath, the bichromate will diffuse out from the tissues into the bichloride, which must be changed every day; at the end of the reaction the preparations will be found decolourised and offering the aspect of fresh tissue. They may be left in the bichloride for any time.

Before mounting, the sections that have been cut must be repeatedly washed with water (if it be wished to mount them permanently), otherwise they will be spoilt by the formation of a black precipitate. Mount in balsam or glycerin; the latter seems the better preservative medium.

The result of this process is not a true stain, but an "apparently black reaction;" the tissues appearing black by transmitted light, *white* by reflected light. Golgi thinks that there is formed in the tissue elements a precipitate of some substance that renders them *opaque*. The elements acted on are (1) the ganglion-cells, with all their processes and ramifications of the processes. These are made more evident than by any other process except the bichromate and silver-nitrate process. An advantage of the mercury process is that it demonstrates nuclei, which is not the case with the silver process. (2) Connective-tissue corpuscles in their characteristic radiate form. But the reaction in this case is far less precise and complete than that obtained by the silver-process. (3) The blood-vessels and particularly their muscular-fibre cells.

The method gives *good* results only with the cortex of the cerebral convolutions, hardly any results at all with the spinal cord, and very scanty results with the cerebellum. And, on the whole, the method shows nothing more than can be demonstrated by the silver-nitrate method, but it is superior to it as regards two points: the reaction can always be obtained with perfect *certainty* in a certain time; and the preparations can be perfectly preserved by the usual methods.

567. Xylol, for Central Nervous System (*Merkel's method* [1]).—Sections from the central nervous system are dehydrated with alcohol of about 94 per cent. (in which they must remain for at least ten minutes), and then cleared with xylol, in which they are examined.

Certain elements of the tissues retain more obstinately than others the small quantity of water that they bring with them out of the 94 per cent. alcohol. Now, as xylol is absolutely immiscible with water, it can exercise no clearing action on these, and they stand out boldly in the picture by virtue of the difference between the index of refraction of their contained water and that of the xylol. The axis-cylinders are at first all that is visible; after a time the ganglion-cells appear with their processes. Nuclei, vessels, and whatever else may be in the preparation, are totally invisible. Merkel thinks this method superior to all others for the study of the distribution of nerve-fibres. The preparations are not permanent, though they may be kept for some weeks by mounting them in Canada balsam. When a preparation (either in xylol or balsam) has become so transparent as to be of no further use (which will always eventually happen), it may be reprepared by putting it back into the alcohol, and thence again into the xylol.

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[1] 'Arch. Mik. Anat.,' xiv (1877), p. 622.

568. Pyroligneous Acid, for Isolating Nerve-Fibres (*Stilling's method*[1]).—Hardened portions of brain are treated with (raw or rectified) pyroligneous acid, and teased out under water. Specimens may then be stained, if desired, with picrocarmine and cleared with oil of cloves.

569. Spinal Cord, Fibres of (*Schiefferdecker's methods*[2]).—A portion of fresh cord has its membranes removed, and is placed for about four weeks in Müller's solution, then washed out for about twenty-four hours with water and brought into alcohol. The sections are washed in water for one or two days, and stained in palladium chloride or gold chloride, washed with water and passed through absolute alcohol and oil of cloves into balsam.

Palladium chloride.—The palladium is used for the demonstration of the longitudinal fibres (and, of course, therefore for staining *longitudinal* sections). A solution of 1·10,000 strength is taken; the sections remain in it till light brown (about three to five hours).

Gold chloride.—The gold chloride is used for demonstrating fine networks in *transverse* sections. Strength 1·5000 or 10,000. Time about one to three hours. After washing in water the sections are put for twenty-four hours into acetic acid of ½ to 1 per cent. then mounted as described above.

570. Spinal Cord (Picro-carminate of soda and palladium chloride) (*Schiefferdecker's methods*[3]).—Sections are placed for one to two minutes in a 1·300 or 600 solution of chloride of palladium, rinsed in water, and thrown for eight or ten minutes into a cold saturated solution of picro-carminate of soda. Mount in dammar.

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[1] Ibid., xviii (1880), p. 471.

[2] Ibid., x (1874), p. 472.

[3] Ibid., xv (1878), p. 38.

For the staining of the isolated ganglion-cells, the following method is employed:—Pieces of spinal cord about half a centimetre thick are macerated in a small quantity (just enough to cover them) of Ranvier's alcohol (one third alcohol) for several days. Small fragments of the grey matter are then taken and well shaken in a test-tube with a small quantity of water. There is then added a little glycerin and a few drops of the concentrated solution of picro-carminate of soda, and the whole set aside for one or two days. Decant, and to the red deposit, which now consists chiefly of stained ganglion-cells, add 1 or 2 drops of glycerin, and place the whole for two days in a desiccator with sulphuric acid. (This part of the operation is best performed in a watch-glass, or, better, flat-bottomed cell.) The cells are best got on to a slide by pouring a drop of the dehydrated glycerin on to it.

No directions are given for the preparation of the picro-carminate; that used by Schiefferdecker was prepared by Dr Witte, of Rostock.

571. Anilin-Blue, for Processes of Ganglion-cells (*Zuppinger's method*[1]).—Sections of brain or cord (that has been hardened in bichromate) are washed out with acidulated water, stained (*in the dark*) in a slightly acidulated (HCl, or acetic acid) solution of the soluble anilin-blue of commerce, washed out with acidulated water, rinsed *quickly* with absolute alcohol, cleared (*in the dark*) with kreasote, and mounted in dammar. They must not be allowed to remain long in the kreasote, and they must be preserved permanently in the dark.

572. Myelon, Sections of, Carmine and Anilin-Blue Double-stain (*Duval's method*[2]).—Stain with carmine, dehydrate with alcohol; stain for five to twenty minutes in an alcoholic solutine of anilin-blue (anilin-blue soluble only in alcohol); do not wash with alcohol, clear with turpentine, mount in balsam or dammar. Duval recommends for the solution of anilin-blue the proportion of 10 drops of a saturated solution to 10 grammes of absolute alcohol, and an immersion of ten to twelve minutes. A violet stain, in which different tissues are very sharply differentiated by the predominance of the red or the blue element; blood-vessels come out with great distinctness, the preparations, though deeply coloured, remain very transparent, and the definition of their elements is remarkably good.

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[1] Ibid., x (1874), p. 255.

[2] Robin's 'Journ. de l'Anat.,' 1876, p. 111.

573. Ganglion-cells, Isolation of (*Carrière's methods*[1]).—Carrière put sections of fresh spinal cord into the three following solutions:

a. Bichromate of potash 1·600.
b. Bichromate of potash 1·500.
c. Chromate of ammonia 1·600.

After ten days the sections were removed from *a* and *b*, washed with water, and thrown into a barely-transparent ammoniacal solution of carmine. Five days afterwards the sections from *a* were found to be in a fit state of maceration for teasing out, but in the sections from *b* the neuroglia was not found sufficiently broken down, and these sections had to be rejected. The sections in *c* were removed after fourteen days into the carmine solution, and after three days therein were found to be fit for teasing. By these means Carrière was able to demonstrate cases of anastomoses between the cells of the anterior cornua.

574. Cerebellum (*Denissenko's method*[2]).—Portions of cerebellum are hardened for two or three weeks or more in a 2 to 5 per cent. solution of bichromate of ammonia, or in Müller's solution. Wash in water for twenty-four hours, and pass first into weak alcohol, then into strong. Sections are then made and stained as follows:—Twenty-four hours in dilute alcoholic solution of eosin, washed with water, and placed in hæmatoxylin until the nuclei become stained. Wash with water and mount in dilute glycerin.

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[1] 'Arch. Mik. Anat.,' xiv (1877), p. 126.

[2] Ibid., p. 205.

This double-stain is said to give a very clear picture, but appears not to be permanent.

575. Fresh Brain, Sections (*Bevan Lewis's method* [1]).—Vertical sections, as thin as possible, are made from a piece of a convolution from which the membranes have been removed. The sections are made by free hand by means of a section-knife flooded with spirit. The sections are got on to a slide and treated with Müller's solution for a few seconds. A cover is then put on and steadily pressed down so as to flatten out the sections into an almost transparent film. The slide is then rinsed in water and placed for thirty to forty seconds in a bath of methylated spirit. It is then removed, one edge of the cover-glass steadied with the finger, the blade of a pen-knife inserted under the opposite edge, and the cover gently lifted. The section, which remains adherent either to the slide or to the cover, is washed with water, stained in any way that may be desired, dehydrated, and mounted in balsam.

For staining the cells of the cortex Lewis prefers a 1 per cent. solution of anilin-black.

He prefers to dehydrate by drying under a bell-glass in the presence of concentrated sulphuric acid. Clear with chloroform in preference to clove oil. Lewis finds that this process results in far less rupture and tearing of nerve-cells and processes than would be imagined; he considers that "the result is equivalent to the most delicate teasing of tissue, the processes being gradually unravelled from their dense networks, and the structural elements universally displayed to the best advantage."

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[1] 'Monthly Micr. Journ.,' vol. xvi (1876), p. 106.

CHAPTER XLI.

CONNECTIVE TISSUES. PLASMA CELLS.

576. Areolar Tissue (*Ranvier's methods*[1]).—Dissociation by means of artificial œdemata is a method that renders great services. The following is an example of its application:—A portion of skin, together with the subcutaneous cellular tissue, is removed by means of a bistoury from a region where fat is not abundant, such as the groin. With a syringe fitted with a sharply-pointed nozzle a small quantity of an appropriate liquid (osmic acid, silver nitrate, picro-carmine, gelatin-solution) is injected. Around the extremity of the nozzle the liquid forms an artificial œdema in the tissues, whose elements are parted from one another and pushed out so as to form a kind of membrane at the surface of the sphere of liquid. By means of curved scissors a segment of the œdematous sphere may easily be removed and brought on to a slide. It must be covered and the cover pressed down *rapidly* as, if time is allowed for the liquid to escape from the network of filaments in which it is held captive, the elements will return to their normal position and the preparation lose all the distinctness that it is the object of this method to obtain.

If nitrate of silver be employed Ranvier recommends a strength of 1·1000; employed in this strength the solution fixes cells without impregnating them, so that subsequent staining with picro-carmine is not interfered with. The method is recommended for the study of fat-cells.

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[1] 'Traité,' p. 329.

577. Adipose Tissue (*Flemming's method*[1]).—Following Ranvier, Flemming recommends the method of artificial œdemata by injection. *Fluid* injection-masses do not suffice. Make a *jelly* of glycerin $\frac{1}{4}$, water $\frac{1}{2}$, gelatin $\frac{1}{4}$; warm it to about 40° C., and mix in about $\frac{1}{10}$th volume of a 5 per cent. silver solution. (This is better than Ranvier's weaker solution.) Inject by puncture, and place the animal to cool on pounded ice. Extract the tumour and place it to *cool* (*not freeze*) on the pounded ice. Cut sections, wash, and expose them for half an hour to sunlight. Stain one hour in picrocarmine. Wash out in water until they appear "transparent rosy-red," rinse in dilute acetic acid (3 to 4 per cent. strength) and mount simply or in glycerin. Formic-acid glycerin (Ranvier) gives *better* results, but the reaction is much slower.

578. Elastic Fibres (*O. Hertwig's methods*[2]).—For the study of the elastic fibres of fibro-cartilage osmic acid is particularly useful. Cartilage treated for one or two hours with a 1 per cent. solution shows the elastic fibres stained of a yellow brown, whilst the matrix remains colourless. Soluble anilin-blue also has an affinity for these fibres. Specimens may have their nuclei stained with carmine, and then be brought for some time into a very dilute solution of anilin-blue; the nuclei will be found to be stained red, the elastic fibres blue, and the matrix will be found colourless. The surest reagent for proving the presence of elastic fibres is solution of potash or soda, which causes cells and matrix to swell up and become transparent, whilst elastic fibres remain unaltered.

579. Cartilage.[3]—Ranvier finds that the following reagents serve to kill cartilage-cells without causing them to shrink:—Picric acid in saturated solution, alum solution of 5·1000, silver nitrate 1·1000, sulphate of copper 1·100, gold chloride 1·200, sodium chloride 1·100, caustic potash 40·100; of all these the alum solution is the best.

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[1] 'Arch. Mik. Anat.,' vii (1872), p. 368.

[2] Ibid., ix (1873), p. 82.

[3] 'Traité,' p. 279.

The best colouring agent is purpurin. It may be combined with the alum solution by adding powdered purpurin to the alum solution and boiling. The solution is filtered hot and 60 grammes of 36° alcohol added for every 200 grammes of the solution.

Sections should be stained in this solution for one or two days, then mounted in glycerin.

580. Bone.[1]—Ranvier points out certain precautions that it is necessary to take in the preparation of sections of dry bone. In general, the bones furnished by "naturalists," or procured in anatomical theatres, contain spots of fatty substance that prevent good preparations from being made. Such spots are formed when bones are allowed to dry before being put into water for maceration; when a bone is left to dry the fat of the medullary canals infiltrates its substance as fast as its water evaporates.

Bones should be plunged into water as soon as the surrounding soft parts have been removed, and should be divided into lengths with a saw whilst wet. The medulla should then be driven out from the central canal by means of a jet of water; spongy bones should be submitted to hydrotomy. This may be done as follows:—An epiphysis having been removed, together with a small portion of the diaphysis, a piece of caoutchouc tubing is fixed by ligature on to the cut end of the diaphysis, and the free end of the piece of tubing adapted to a tap through which water flows under pressure.

As soon as the bones, whether compact or spongy, have been freed from their medullary substance they are put to macerate. The maceration should be continued for several months, the liquid being changed from time to time. As soon as all the soft parts are perfectly destroyed, the bones may be left to dry. When dry, they should be of an ivory whiteness, and their surfaces exposed by cutting of a uniform dulness.

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[1] 'Traité,' p. 297.

Thin sections may then be cut with a saw and prepared by rubbing down with pumice-stone (l. c., p. 82). Compact pumice-stone should be taken and cut in the direction of its fibres. The surface should be moistened with water and the section of bone rubbed down on it with the fingers. When both sides of the sections have been rubbed smooth in this way, another pumice-stone may be taken, the section placed between the two, and the rubbing continued. As soon as the section is thin enough to be almost transparent it is polished by rubbing with water (with the fingers) on a Turkey hone or lithographic stone. Spongy bone should be soaked in gum and dried before rubbing down (but *see* von Koch's copal method, No. 262).

581. Yellow Elastic Tissue (*Stirling's method*[1]).—Make sections of ligamentum nuchæ of ox. Stain picro-carmine.

The elastic fibres are stained yellow, while the small amount of connective-tissue between the elastic fibres becomes red.

582. Yellow Fibro-Cartilage (*Stirling's method*[2]).—Epiglottis of ox. Hardened in saturated picric-acid solution, twenty-four hours. Sections. Stain picro-carmine. (Connective tissue and nuclei of mucous-gland cells, red; perichondrium, red; elastic fibres, yellow; nuclei of cartilage, red.)

Mount in Farrant's medium or glycerin.

583. Fœtal Bone (*Stirling's method*[3]).—Decalcify, picric acid; stain, picro-carmine. (Connective tissue and bone-corpuscles, red; matrix, yellow.)

584. Aorta (*Stirling's methods*[4]).—Picric acid, twenty-four hours; make sections, stain picro-carmine; mount in Farrant's medium. (All connective tissue red, all elastic fibres yellow, smooth muscle yellowish brown.)

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[1] 'Journ. of Anat. and Phys.,' xv (1881), p. 349.

[2] Ibid.

[3] Ibid.

[4] Ibid., p. 351.

585. Plasma-cells. Dahlia and other Anilin Stains (*Ehrlich's methods*[1]).—Dahlia (or monophenylrosanilin) is very nearly related in its composition and its reactions to Parma-blue (diphenylrosanilin) and anilin-blue (triphenylrosanilin). Preparations soluble in water, as well as kinds soluble only in alcohol, are found in commerce. (Ehrlich obtained those used by him from Herrn J. Frank, Apotheker in Freiburg.)

First method.—A neutral aqueous solution of dahlia stains the formed material and the protoplasm of most tissues, whilst leaving the nuclei unstained. If the stained tissues be treated with water acidulated with acetic acid, the stain is washed out from the tissue and protoplasm and taken up by the nuclei, thus giving a stain that resembles that of hæmatoxylin. If any "plasma-cells" be present in the preparations they are brought into evidence at once by the superior intensity with which they stain.

Preparations obtained in this manner should be dehydrated in absolute alcohol and mounted in solution of resin in turpentine.

586. *Second method.*—It may desirable to obtain a *specific* stain of the plasma-cells alone, which may be done as follows:

The tissues must first be well hardened in strong alcohol (chromic acid and its salts must be avoided). They are then placed for at least twelve hours in a staining fluid composed of—

Alcohol absolute	50 c.c.
Aqua	100 c.c.
Acid. acet. glacial.	12½ c.c.

to which has been added enough dahlia to give an almost saturated solution. After staining, the preparations are transferred to alcohol, which washes out the stain from all but the plasma-cells, and may then be mounted in the resin-turpentine solution.

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[1] 'Arch. Mik. Anat.,' xiii (1876), p. 263.

The degree to which the colour is removed by this process of washing out depends on the degree of acidity of the staining fluid. "A solution that contained only $7\frac{1}{2}$ c.c. of glacial acetic acid, yet stained the preparations with moderate intensity." Smaller proportions of acetic acid may therefore be taken for preparations in which there is much connective tissue, but for structures in which cells predominate the more strongly acidulated solutions are to be preferred.

Mucous-cells and fat-cells are also sometimes stained by these solutions.

Other media.—In a similar way other soluble anilins may be employed (in the form of a fluid containing $7\frac{1}{2}$ c.c. of acetic acid),—primula, iodine-violet, methyl-violet, purpurin, saffranin, fuchsin; of these, methyl-violet gives the best results.

As regards the manner in which the plasma-cells are stained by these methods, it remains to be said that the nucleus is uncoloured, the protoplasm stained weakly or not at all, whilst the surrounding granules which form the characteristic element of these cells are stained intensely.

587. Plasma-cells (*Korybutt-Daszkiewicz's methods*[1]).—Frogs were kept for two months (in summer) without food, then placed in a reservoir of running water and well fed for four weeks. "Plasma-cells" were then found in abundance. The nerves were first treated with osmic acid of 1·200, and then stained with a slightly-acidulated ammonia carmine. Fuchsin gives the finest stains, but the colour is not permanent. The "plasma-cells" appear to have a special affinity for anilin colours, especially for dahlia (Ehrlich, 'Arch. Mik. Anat.,' xiii), (*see* Formula No. 585).

The best method for permanent preparations is to stain with dahlia, methyl-violet, fuchsin, or other anilin stains, dehydrate in alcohol, and mount in turpentine. Turpentine in which resin is dissolved is a very useful medium for teasing. It is well to first harden the tissues in osmic acid.

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[1] Ibid., xv (1878), p. 7.

CHAPTER XLII.

BLOOD. GLANDS. LYMPHATICS.

588. Eosin as a Reagent for Hæmoglobin (*Wissozky's methods*[1]).—Equal parts of alum and eosin are dissolved in 200 parts alcohol. If blood be treated with water and then with the eosin staining-solution, and washed out with water, it is found that the red blood-corpuscles stain rose-red in the parts from which the water has not dissolved out the hæmoglobin. The nucleus when existent always remains unstained. It may be then stained with hæmatoxylin. White blood-corpuscles are stained by hæmatoxylin but not by eosin.

589. Blood-corpuscles (*Boettcher's method*[2]).—Make a saturated solution of corrosive sublimate in alcohol of 96 per cent. One volume of blood is thrown into 50 volumes of this solution, well shaken up with it, and allowed to remain there for twenty-four, or, better, forty-eight hours. The corpuscles by this time are freed of their colouring matter. The sublimate-solution is now poured off, and the deposit shaken up with alcohol, in which it remains twenty-four hours, after which the alcohol is poured off and water substituted. The corpuscles are now so hardened that they may remain for days in the water without injury. After washing they may be stained with any agents that are desired in order to demonstrate the "nucleus" or other structural arrangements which Boettcher claims to have made out by this means in mammalian blood.

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[1] 'Arch. Mik. Anat.,' xiii (1876), p. 479.

[2] Ibid., xiv (1877), p. 74.

590. Osmium Vapour for Blood-corpuscles (*Dr. Schmidt's formula*[1]).—Expose a drop of blood to the vapour of a 2 per cent. osmic-acid solution for two or three minutes and mount in glycerin. If acetate of potash be used for mounting it will render the corpuscles indistinct in a very short time.

591. Blood of Birds (Methyl-Violet) (*Bizzozero and Torre's method*[2]).—Dilute a drop of blood with 0·75 per cent. salt-solution in which has been dissolved a little *methyl-violet.* This liquid in nowise affects the form of the elements, stains intensely the nucleus of the red corpuscles, and, in the white, stains the nucleus intensely, and the protoplasm less intensely. May be used for the study of bone-marrow and spleen.

592. Bone-marrow (*Bizzozero and Torre's method*[3]).—Müller's solution three days, then *hang up in* a cylinder of commercial alcohol, which change frequently till it remains colourless. Water ten minutes, then gum-solution twenty-four hours; commercial alcohol slightly diluted with water until the gum is hardened. Sections: picro-carmine (on the slide) two hours; glycerin. (It is important *not* to use water for washing out the picro-carmine as that would deprive the red blood-corpuscles of their yellow stain.)

593. Blood (*Stirling's methods*[4]).—Osmic acid (1 per cent.) five minutes, picro-carmine with a trace of glycerin three or four hours. For blood and epithelium-cells.

Picric acid, saturated solution, five minutes; picro-carmine with a trace of glycerin, one hour. Mount in Farrant's medium or glycerin. For blood.

(In both these cases the operation is carried out on the slide by mixing a drop of blood with a drop of the fixing solution, which is subsequently removed with blotting-paper in the usual way.)

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[1] 'Journ. Roy. Mic. Soc.,' i (1878), p. 75.

[2] 'Archivio per le scienze mediche,' vol. iv, No. 18 (1880), p. 390.

[3] Ibid., p. 405.

[4] 'Journ. of Anat. and Phys.,' xv (1881), p. 349.

594. Kidney (*Tubuli contorti*) (*Heidenhain's methods*[2]).—After examination of sections of fresh kidney, both without addition of any liquid and with addition of water, salt-solution, or dilute acids, sections of the hardened kidney should be examined. Small pieces of perfectly fresh kidney are hardened in absolute alcohol (it is well to first inject the still warm kidney with absolute alcohol from the renal artery or vein).

Sections of the hardened kidney are treated with glycerin, or, which is better, with HCl of 0·1 per cent. The bacilli of the bacillary epithelium swell up, and are most instructively brought out by this method. Instead of alcohol and HCl in succession a strongly acid mixture of alcohol and acetic acid may be used for hardening. Staining should be avoided.

Before hardening in alcohol the kidney may advantageously be treated for twenty-four hours with a 5 per cent. solution of neutral chromate of ammonia, after which they must be thoroughly washed out with water before coming into the alcohol.

Another excellent method of demonstrating the bacilli is to inject from the renal artery or vein a cold saturated solution of potassium chloride, harden portions in alcohol, make sections, clear with turpentine, and mount in dammar.

To make isolation-preparations of the tubuli, maceration must be resorted to. Pieces of kidney may be laid for several hours in nitric acid, either concentrated (but not fuming) or diluted with two vols. water; they are afterwards preserved in dilute glycerin (glycerin 2 parts, water 1 part.) Nitric acid is better than hydrochloric, as it preserves the forms of the different epithelia; the bacilli are well brought out.

Isolation of the bacilli themselves may be obtained by maceration in caustic soda (33 per cent.), molybdate of ammonia (2½ to 5 per cent.), and above all, in the above-men-

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[1] 'Arch. Mik. Anat.,' x (1874), p. 6 ff.

tioned 5 per cent. solution of neutral chromate of ammonia. The tissues should remain in this solution for twenty-four hours, and it is well, before teasing, to wash them out with water for twenty-four or forty-eight hours. They may, if desired, remain in the solution for months without becoming brittle (as is the case when the bichromate or Müller's solution is employed).

595. Liver, Stellate Cells of (*Kupffer's method*[1]).—Sections are cut from a fresh liver by means of a Valentin's knife, washed for fifteen minutes with 0·6 per cent. salt-solution, or (better) with 0·05 per cent. chromic acid, then brought into a solution of gold chloride 1 part, HCl 1 part, water 10,000 parts; and left there in the dark until the gold is reduced, which is generally the case after forty-eight hours. Mount in acidulated glycerin.

596. Salivary Glands (*Lavdowsky's methods*[2]).—*Chloral.*—Hydrate of chloral, which in 5 per cent. solution forms an excellent preservative liquid, is strongly recommended for the study of smooth muscle-fibre in general, and especially for that of salivary glands. A small portion of a fresh gland is macerated for twenty to twenty-four hours in as large a quantity as possible of 5 per cent. chloral hydrate, and then teased out in the same liquid.

Alcohol.—Contrary to the opinion of Ranvier, absolute alcohol is a most valuable reagent for hardening these and similar organs (glands of stomach and intestine), provided that the peripheral portions of the organ, which are caused to shrink by the too energetic action of the alcohol, be rejected, and sections from the *centre* of the gland selected for study.

For staining: 1. Picro-carmine. 2. Picro-carmine, followed by hæmatoxylin. 3. Ammonia-eosin. 4. Picro-eosin. 5. Chloride of gold, 0·3 to 0·5 per cent., with subsequent treatment by ammonium sulphide, or by dilute alcohol (3 to 4 parts water and 1 of absolute alcohol). 6. Osmic acid, ½ to 1 per cent. 7. Ammonia-carmine. 8. Hæmatoxylin. The neutral (single) chromate of ammonia is used in 5 per cent. solution; chromic acid in 1 to 30,000.

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[1] Ibid., xii (1875), p. 353.

[2] Ibid., xiii (1876), p. 359

597. Endothelium of Lymph-spaces of the Eye.[1]—*Schwalbe* recommends treatment for *half a minute* with *¼ per cent.* silver solution.

In an excursus on the silver-method, Schwalbe concludes, after reviewing the evidence, that the silver is reduced in a layer of liquid that exists in the superficial furrows between contiguous cells; the black lines thus formed are a true precipitate, and this kind of stain must be distinguished from the other reaction in which by exposing tissues for some time to the action of more concentrated solutions, there is formed a combination of the inter-cellular cement-substance with the silver, that turns *brown* on exposure to light.

598. Lymphatics of Skin, Gold- and Silver-method (*the Hoggans' method*[2]).—A piece of skin having been stretched on the authors' histological rings[3] (see description of the apparatus, l. c., also 'Journ. Roy. Mic. Soc.,' vol. ii, p. 357), the hypodermis is treated for 30 seconds with a ½ per cent. solution of silver nitrate, then washed with water and treated for 30 seconds with a ½ per cent. solution of gold chloride; then washed with water and exposed for a short time to diffused daylight. Treat with glycerin (whilst still on the rings), remove the hair (and epidermis if possible), and mount in glycerin. Avoid acetic acid.

599. Lymphatics of Bladder, Gold- and Silver-method (*the*

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[1] Ibid., vi (1870), p. 5.

[2] Robin's 'Journ. de l'Anatomie,' &c., 1879, p. 54.

[3] The Hoggans' histological rings are supplied by Burge and Warren, 42, Kirby Street, Hatton Garden, E.C., at 10*s*. per dozen pairs, prepaid (1 inch inside diameter).

Hoggans' method[1]).—The bladder, stretched on the *Hoggans'* histological rings, with the epithelial surface upwards, is treated as follows (the epithelium having been first scraped off). Nitrate of silver, 1 to 2 per cent., quickly poured on and off; expose to dull light for a few minutes; wash; gold chloride, 1 per cent., 1 minute; wash; reduce in (diffused?) light; clear glycerin or oil of cloves; excise from the rings, and mount.

600. Lymphatics of Pancreas (*the Hoggans' method*[2]).—A portion of the diffused pancreas of a small rodent is stretched on histological rings and treated as follows. Silver nitrate, 1 per cent., quickly poured on and off; wash; reduce (excessive exposure is necessary). Stain hematoxylin, wash, dehydrate, clear (oil of cloves), mount in "varnish."

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[1] 'Journ. of Anat. and Phys.,' xv (1881), p. 359.

[2] Ibid., p. 477

CHAPTER XLIII.

MOLLUSCA.

601. Ciliated Epithelium (Intestine of Anodonta) (*Marchi's methods* [1]).—Maceration and teasing in bichromate of potash ($\frac{1}{2}$ to 1 per cent.), osmic acid ($\frac{1}{2}$ per cent.), and iodized serum.

602. Limax, Mucus-cells of Epidermis (*Marchi's methods* [2]).—If a living Limax be thrown into moderately concentrated salt-solution it will throw off enormous quantities of mucus, and die in a few hours. The epidermis will be found well preserved. If the animal be thrown into osmic acid or Müller's solution, if I understand the writer justly, no secretion of mucus will occur. For hardening, osmic acid or Müller's solution; for maceration, 1 per cent. bichromate of potash.

603. Histology of Mollusca (*Boll's methods* [3]).—For isolation of muscle-fibres, teasing in humor aqueus, &c., then potash solution of 33 per cent. then maceration in 1 to 2 per cent. bichromate of potash.

For epidermis, maceration in 1 per cent. bichromate, or in cold-saturated solution of oxalic acid, or in the latter mixed with an equal volume of iodized serum to reduce the too great energy with which the pure acid macerates, or in iodized serum.

For hardening epidermis, osmic acid of 1 to 2 per cent. is the best; twenty-four to forty-eight hours in this solution generally suffice. Alcohol deforms the cells. Bichromate of potash is excellent, but very slow in its action; *strong* solutions must be taken, since up to 2 per cent. its action is to macerate the epidermis (of Mollusca).

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[1] 'Arch. Mik. Anat.,' ii (1866), p. 468.

[2] Ibid., iii (1867), p. 204.

[3] Ibid., v (1869), passim (separate pagination).

604. Epidermis of Mollusca (*Flemming's method*[1]).—For the isolation of the setiferous sense-cells maceration for several days in bichromate of potash of 4 to 6 per cent., which is better than the 1 per cent. solution recommended by Boll. Relatively *large* pieces of tissue should be taken, and placed in relatively small quantities of the liquid, in order to obtain the production of a desirable proportion of colloid and crystalloid substances. An entire mussel may be macerated for four to eight days in about three ounces of the liquid.

605. Gasteropoda, Tentacles of (*Flemming's methods*[2]).—The first difficulty here is to obtain the excision of an exserted eye. It is impossible to sever the exserted peduncle in a living animal without its retracting at least partially before the cut is completed. Never mind that; make a rapid cut at the base, and throw the organ into very dilute chromic acid, or 4 per cent. bichromate; after a short time it will evaginate, and remain as completely erect as if alive. Harden in 1 per cent. osmium, in alcohol, or in bichromate.

606. Gold-process for Mytilus edulis (Nerves of Mantle) (*Flemming's method*[3]).—HCl (1 per cent.) one hour, gold-solution ($\frac{1}{4}$ per cent.) twelve hours, water acidulated with ac. acet. several days, even up to ten days, in the sunlight. Harden in alcohol afterwards if necessary.

607. Mollusca (Acephala) Injection of (*Flemming's method*[4]) —To kill the animals freeze them in a salt-and-ice mixture, and throw them for half an hour into lukewarm water. They will be found dead, and in a fit state for injection. Chloroform and ether are useless. The injection-pipe may be tied in the heart; but when this has been accomplished there remains the problem of occluding cut vessels that it is impossible to tie. To this end, after the pipe has been tied, the entire animal is filled and covered up with plaster of Paris. As soon as the plaster has hardened, the injection may be proceeded with.

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[1] Ibid., p. 419.
[2] Ibid., vi (1870), p. 441.
[3] Ibid., p. 454.
[4] Ibid., xv (1878), p. 252.

608. Cilia and Ciliated Epithelium (*Engelmann's methods*[1]).—Engelmann strongly recommends the use of green light for delicate observations as giving sharper definition, allowing finer detail to be seen, and tiring the eyes less than white light. Green glass of sufficiently good quality is found in commerce. The glass is best put between the mirror and the object, *e.g.* on the diaphragm. Blue glass (cobalt or ammonio-sulphate of copper) is also useful, but less so than green. Red light is most hurtful. "The explanation of these points, so important in practice, may be found in the results obtained by Lamansky in his researches on the "Limits of sensibility of the eye to the different colours of the spectrum," 'Arch. f. Ophthalm.,' xvii, p. 123, 1871. (It is to be presumed that Engelmann means to recommend the combination of green glass with white daylight, in order to obtain green light; if lamplight be used, a blue glass of the proper tint will give green light. I am in the habit of using a glass globe filled with ammonio-sulphate of copper, which I place between the lamp and the mirror.)

Carchesium polypinum.—Living, or in ½ per cent. osmic acid.

Cyclas, Rana.—For demonstration of the bacillar layer, maceration in one-third alcohol, Müller's solution, boracic or salicylic acid.

Cyclas cornea (intestine), maceration in osmic acid of 0·2 per cent. (after having warmed the animal for a short time to 45° to 50° C.). Also, concentrated boracic-acid solution.

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[1] 'Pfluger's Archiv,' xxiii (1880), p. 505, *et seq.*

The intra-cellular processes of the cilia.—The entire intracellular fibre-apparatus may be *isolated* by teasing fresh epithelium from the intestine of a Lamellibranch (*e. g.* Anodonta) in either bichromate of potash of 4 per cent., or salt-solution of 10 per cent. To get good views of the apparatus *in situ* in the body of the cell, macerate for not more than an hour in concentrated solution of boracic or salicylic acid. Very dilute osmic acid (*e. g.* 0·1 per cent.) gives also good results. The "lateral cells" of the gills are best treated with strong boracic-acid solution (5 parts cold saturated aqueous solution to 1 part water).

For Vertebrata, the above-mentioned reagents are not successful. For these, maceration for twenty-four hours in one-third alcohol, or for a day in Müller's solution, is recommended.

609. Terrestrial Pulmonata. Foot-glands (*Sochaczewer's method*[1]).—Portions of the foot, containing the foot-glands, are hardened in $\frac{1}{2}$ per cent. osmic acid. The excess of acid is rinsed away and the specimens placed for from four to five days in chromic acid of 1 per cent., or bichromate of potash of 4 to 6 per cent. They are then washed out with a mixture of glycerin, water, and alcohol, and brought into absolute alcohol. This gives preparations of the consistence of cartilage, and with a Rivet-Leiser microtome sections of $\frac{1}{40}$ to $\frac{1}{80}$ mm., may be cut. They are best double-stained with picro-carmine and hæmatoxylin.

For maceration, put for three or four days into osmic acid of $\frac{1}{20}$ to $\frac{1}{50}$ per cent., or for five to six days into $\frac{1}{10}$ per cent. chromic acid.

610. Lamellibranchiata. Pallial Nerves (*Vialleton's method*[2])—A fresh piece of the mantle is treated as follows: Lemon-juice, fifteen minutes; gold chloride, 1 per cent., twenty minutes; acidulated water (1 drop of acetic acid to 20 grammes of water), twenty-four to thirty-six hours.

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[1] 'Zeit. wiss. Zool.,' xxxv (1881), p. 40.

[2] 'Comptes rendus,' 1882 (2me série), p. 461.

611. Mollusca (Prosobranchiata). Foot-glands (*J. Carrière's method*[1]).—Chromic acid, $\frac{1}{3}$ per cent., six to eight hours; alcohol, 50 per cent., 70 per cent. Clear with oil of cloves, imbed in paraffin, using the air-pump. Stain, picro-carmine, fuchsin, or cochineal; double stain, picro-carmine and cochineal.

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[1] 'Arch. Mik. Anat.,' xxi (1882), p. 388.

CHAPTER XLIV.

ARTHROPODA.

612. Nerve and Muscle of Arctiscoida (*Doyère's method*[1]).—A score or so of *Milnesium tardigradum* are collected (it is well to have a large number, as the process by no means succeeds with all individuals) and put into a test-tube with water that has been deprived of its air by boiling. A drop of oil is run on to the surface of the water, so as thoroughly to exclude the air. After twenty-four to forty-eight hours the animals will be found, not dead, but fixed and extended in a cataleptic state; the circulation of the perivisceral fluid has ceased, the pigment of the cuticle has disappeared or collected into patches that are no hindrance to observation, the entire animal has gained in transparency, and the nervous and muscular systems stand boldly out. The animals are examined in boiled water, unless it be wished to study the phenomena of resuscitation, in which case spring-water should be used.

613. Brain of Insects (*Wagner's method*[2]).—Harden in Betz's liquid (mixture of equal parts of sulphuric ether and chloroform), and make sections.

614. Striated Muscle (*Thanhoffer's method*[3]).—In order to demonstrate the two plates of the sarcolemma, digest muscle

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[1] 'Arch. Mik. Anat.,' i (1865), p. 105.

[2] 'Comptes rendus,' 1879 (2me série), p. 379.

[3] 'Arch. Mik. Anat.,' xxi (1882), p. 27.

(of an insect) either in the stomach of a living animal (by wrapping it in gauze and introducing it through a fistula) or in artificial gastric juice (in the former case several hours, in the latter half to one hour, at the temperature of the room in summer).

615. Crayfish (Nervous Centres) (*Krieger's methods*[1]).—For the study of nuclei and protoplasm of ganglion-cells, *osmic acid* (applied in the shape of vapour until the cells begin to turn brown), followed by staining for twelve hours in picro-carmine. After this macerate in very dilute picro-carmine to which a little picric acid has been added.

For the study of ganglion-cell processes macerate for three or four days in solution of chromic acid or chromate of ammonia of 2 to 0·005 per cent.

Sections.—For hardening, chromic acid of $\frac{1}{3}$ per cent. (Alcohol causes shrinking; even osmic acid causes shrinking of the larger nerve-fibres, and so does picro-sulphuric acid.) The tissues should remain in the chromic acid for twenty-four hours. Wash out for twenty-four hours in water frequently changed. Preserve in alcohol. Stain before cutting in acetic-acid carmine or picro-carmine. The acid carmine gives the more brilliant stain, but has not penetrating power enough for the larger ganglia, for which picro-carmine is very suitable. Wash with water, dehydrate, clear with clove oil, imbed in paraffin, cut (with a Rivet-Leiser microtome).

616. Arachnida (Phalangida) (*Rössler's method*[2]).—The animals are killed in boiling water; the water is allowed to boil up several times, so that the albumen of the tissues may be coagulated; they are then brought into alcohol, first of 70, then 90 per cent., then absolute, until all water is removed from them. They are then imbedded in soap. The soap is remelted, and allowed to cool once or twice, in order to get the objects thoroughly penetrated. Sections are then made, and stained on the slide with some colouring matter dissolved in absolute alcohol.

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[1] 'Zeit. wiss. Zool.,' xxiii, p. 529.

[2] Ibid., xxxvi (1882), p. 672.

Paraffin was tried for imbedding, but gave no good results, on account of the brittleness of the tissues caused by the preliminary treatment with turpentine or oil of cloves.

617. Arthropoda in General (*Mayer's methods*).—It may safely be stated that, as general methods for the study of chitinous structures, the methods worked out by Paul Mayer are superior to all others. It is absolutely necessary that all processes of fixation, washing, and staining should be done with fluids possessing great penetrating power. Hence, picric-acid combinations should be used for fixing, and alcoholic fluids for washing and staining. *Concentrated* picro-sulphuric acid is the most generally useful fixative, 70 per cent. alcohol is the most useful strength for washing out, and tincture of cochineal in alcohol of 70 per cent. is the most generally useful staining fluid. *See* **the Naples Methods,** No. 3; for picric-acid solutions, *see* Nos. 22 to 26; and for cochineal, *see* No. 87. Kleinenberg's hæmatoxylin may sometimes be preferable, *see* Nos. 91 and 92. For alcoholic carmine and borax-carmine, *see* Nos. 84, 85, 81, and 82.

CHAPTER XLV.

VERMES.

618. Vermes, Hardening of, in Clove Oil[1].—For the larger *worms* Rindfleisch states that if stained and cleared *in toto* in oil of cloves they will acquire a fit degree of hardness for section-cutting.

619. Annelida (Magelona) (*W. C. McIntosh's method*[2]).—The animals are thrown into absolute alcohol, pinned on cork, put back together with the cork into alcohol, and cut into sections with the razor and the free hand. The sections are mounted in chloride of calcium.

This simple plan was found to answer just as well as more complicated methods (November, 1878).

620. Trichinæ (*Tikhomiroff's method*[3]).—Digest small pieces of pork for half an hour with their weight of chlorate of potash, to which is added four times as much nitric acid. Agitate with water until the fibrils separate. (For searching for trichinæ.)

621. Nerve-elements of Invertebrates (*H. Schultze's methods*[4]).—*Mollusca.*—Iodized serum, body-lymph of the animal, ammonia bichromate of 0·02 per cent. or weaker, osmic acid, alcohol of 20 to 10 per cent. (these three last as preservative agents). Gold chloride and formic acid, concentrated acetic acid followed by carmine, picro-carminate of soda, hæmatoxylin.

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[1] 'Arch. Mik. Anat.,' i (1865), p. 138.

[2] 'Zeit. wiss. Zool.,' xxxi, p. 412.

[3] 'Journ. Roy. Mic. Soc.' i (1878), p. 26. From 'La Nature,' Feb. 2, 1878.

[4] 'Arch. Mik. Anat.,' xvi (1879), p. 66.

Vermes.—Blood of the animal, iodized serum. Place the nerve-cord for some seconds in concentrated acetic acid, or for a longer time in sulphuric acid (in order to macerate the very resistent neurilemma); harden in weak ammonia bichromate.

622. Annelida (Ciliated Epithelium) (*Gaule's methods*[1]).—The object was *Aricia fœtida*, whose gills possess on either side a row of massive and powerful cilia. The *Ariciæ* were fixed with osmic acid, concentrated solution of corrosive sublimate, picro-sulphuric acid, solutio Mülleri.

Corrosive sublimate is preferable to the other liquids in respect of the perfection of staining that may be attained after its action. For staining were used, Grenacher's carmine (*which* carmine?), hæmatoxylin, or aqueous solution of anilin-blue.

623. Platyhelmia (*Lang's methods*[2]).—For fixing, Lang always uses one of the corrosive sublimate solutions, No. 30, and generally the 3rd or the 2nd formula in preference to the more complicated original solution. Wash out as directed, and stain with the picro-carmine and eosin as directed, No. 71. Imbed in paraffin; it is recommended that kreasote be copiously employed.

Lang finds that this process is *the only one* by which the histology of *Dendrocœla* can be successfully studied.

624. Hirudinea (Nephridia of the) (*O. Schultze's methods*[3]).—For isolation of the Nephridia, dissection of fresh material; or treatment for six hours with 20 per cent. nitric acid (followed or not by maceration in water for one to three days).

For sections: harden for one day in 0·2 per cent. chromic acid (inject the acid from the mouth after having ligatured the anus), wash out in water for several hours, and bring into alcohol gradually increased in concentration up to 98 per cent. Stain for twenty-four hours in hæmatoxylin, and wash out in 0·5 per cent. alum-solution.

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[1] 'Arch. Anat. u. Phys.' (Phys. Abth.), 1881, p. 155.

[2] 'Zool. Anz.,' No. 19 (1879).

[3] 'Arch. Mik. Anat.,' xxii (1883), p. 78.

CHAPTER XLVI.

CŒLENTERATA. PORIFERA.

625. Medusæ (*O. and R. Hertwig's methods*[1]).—The animals are killed in very dilute osmic acid, which the authors prefer to all other reagents for transparent pelagic animals. The acid should be allowed to act only for a few minutes.

626. Macerating Fluids for Medusæ.[2]—Solutions of osmic acid of 0·05 per cent. allowed to act for three minutes suffice to harden the cells, and subsequent soaking in glycerin affords a useful degree of maceration.

A better process is the following:—The objects are treated for two or three minutes, according to their size, with a mixture of equal volumes of 0·2 per cent. acetic acid and 0·05 per cent. osmic acid, and then washed in repeated changes of 0·1 per cent. acetic acid until all traces of free osmic acid are removed. They then remain for a day in 0·1 per cent. acetic acid, are then washed with pure water, stained with Beale's carmine, and preserved in glycerin.

Amongst other advantages of this mixture it is noted that the reduction of osmic acid by albuminates is greatly hastened by the presence of acetic acid, which in the case of animals so transparent and poor in cells as medusæ is an advantage for the study of the nervous system. For ganglion-cells and nerve-fibrils reduce osmium quicker than common epithelium-cells. They become greenish brown, and are easily distinguished from surrounding tissues.

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[1] 'Das Nervensystem u. die Sinnesorgane der Medusen,' Leipzig, 1878, p. 4.

[2] Ibid., p. 5.

The isolation of the elements of the macerated tissues is best done by gently tapping the cover-glass (which may be supported on wax feet.—AUTHOR). This gives far better results than teasing with needles. A camel's-hair pencil also sometimes renders good service.

For hardening, medusæ were treated for five to fifteen minutes with 0·5 per cent. osmic acid, stained with dilute Beale's carmine or picro-carmine, and preserved in weak alcohol. The carmine staining is necessary in order to prevent the osmium from blackening.

627. Medusæ. Imbedding Methods (*O. and R. Hertwig's method*[1]).—A piece of liver hardened in alcohol is cut in two, a hollow answering to the size of the object to be imbedded is scooped out on the flat surface of each of the pieces of liver, and is filled with dilute gum-glycerin. The object is fixed in position in the hollow with needles, the two halves of the liver are replaced in their natural position, and the whole is put into dilute spirit until the gum is sufficiently hardened for cutting.

628. Corals.—Von Koch's method of preparing sections of corals, in which both the hard and the soft parts are preserved *in sitû,* has been given under No. 262 (IMBEDDING METHODS).

629. Medusæ. Salt and Alum as Fixing Agents (*Pagenstecher's formula*[2]).—Take 2 parts common salt and 1 part alum, and make a strong solution. The addition of corrosive sublimate (Goadby) is better avoided. Throw the animals alive into the solution, and leave them there for twenty-four to forty-eight hours. Afterwards preserve in weak alcohol, which must be changed if it becomes cloudy. (For Medusæ.)

630. Alum Sea-water.—Saturated solution of *alum in sea-*

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[1] Ibid., p. 6.

[2] 'Zeit. wiss. Zool.,' xvii (1867), p. 379.

water is largely used at the Villefranche Zoological Station as a fixative and preservative for *Ctenophora*, *Siphonophora*, and other Cœlenterates, as well as for many other delicate pelagic organisms.

631. Porifera (*Dezsö's method*[1]).—Specimens of *Tethya lyncurium* (gemmæ) preserved in absolute alcohol were treated with 0·25 per cent. osmic acid, stained with neutral carmine solution (Beale's formula), cleared, and imbedded in paraffin for cutting. As regards skeletal parts, it should be noted that Dezsö found that the *small* stellate spicula of the cortex are completely dissolved by boiling hydrochloric acid. It seems to the present writer that the study of the skeleton of siliceous sponges should always be controlled by sections mounted in balsam *without being* treated with either acids or alkalies. And it should further be noted that such sections should *not* be mounted in glycerin-jelly, which may happen to have the same refractive index as the silex of the spicula, in which case the latter will become perfectly invisible. I have lost several slides in this way. Gum-water produces the same effect.

632. Porifera (Reniera semitubulosa) (*Keller's methods*[2]).—The living sponge was treated with sea-water, gold and silver salts, and osmic acid of from $\frac{1}{20}$th to $\frac{1}{10}$th per cent. (The commonly employed 1 per cent. solution is *much too strong*.) Alcohol specimens were treated with picro-carmine, eosin, and ammonia-carmine. The best-preserved specimens were obtained by means of osmic acid followed by weak chromic acid. It is essential to the success of the silver-process for demonstrating the pavement epithelium of the ectoderm that the surface of the sponge be *perfectly clean*.

633. Porifera (Embryology) (*F. E. Schultze's methods*[3]).—For the study of the larvæ of *Sycandra raphanus*, Schultze employed (besides the observation of the living larvæ in hanging-drop moist chambers, oxygenated by means of a few fronds of green algæ), osmic acid followed by picro-carmine.

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[1] 'Arch. Mik. Anat.,' xvi (1879), p. 627.

[2] 'Zeit. wiss. Zool.,' xxx, p. 565.

[3] Ibid., xxxi, p. 295.

634. Gymnoblastic Hydroids (Tubularia mesembryanthemum) (*Ciamician's methods* [1]).—For general observation of the tissues, osmic acid of $\frac{1}{4}$ to $\frac{1}{2}$ per cent. For dissociation of the tissue-elements, macerate in 1 per cent chromic acid, and tease in dilute glycerin. It is advantageous to stain with eosin in a very dilute solution before teasing.

635. Porifera (Ova of Chalina) (*Keller's method* [2]).—Segmentating ova are dehydrated in absolute alcohol, and then cleared for examination *in toto* by placing them for two or three days in a not too thick solution of sandarak, in which they are then examined.

636. Porifera (Larvæ of Plakina) (*F. E. Schultze's method* [3]).—Blastulæ were hardened in osmic acid or absolute alcohol, stained *in toto* with hæmatoxylin, picro-carmine, or alum-carmine; dehydrated completely, imbedded in paraffin, and cut into sections with a Leiser microtome.

The best sections of the more advanced *sessile* larvæ were obtained by selecting larvæ that had settled down on thin fronds of algæ and treating them, together with the fronds, with osmic acid, then alcohol of 52 per cent., alum-carmine, Aq. dest., alcohol of 52 per cent., then 70 per cent., 95 per cent., then absolute alcohol, turpentine, and finally paraffin.

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[1] Ibid., xxxii, p. 323.

[2] Ibid., xxxiii (1879), p. 333.

[3] Ibid., xxxiv (1880), p. 416.

CHAPTER XLVII.

PROTISTA.

637. Osmic Acid for Infusoria, &c. (*Pelletan's formulæ*[1]).—Drop a little 1 per cent. osmic-acid solution on the organisms and expose to a current of air, which carries off the vapours of osmium and evaporates the water. Mount in 1 per cent. carbolic-acid solution. If it be desired to stain, use a 1 to 400 or 500 solution of gold chloride, placing the preparations in the light and washing with distilled water. Overstained preparations may be treated with very dilute formic acid, or may be mounted in glycerin.

638. Infusoria (*Certes' methods*[2]).—Fix with osmic acid of 2 per cent. (In the case of very contractile Infusoria, place a drop of the solution on the cover-glass, and place it on the drop of water that contains them. But generally speaking it is best to employ only the vapour of the solution, exposing the organisms to its action for not more than from ten to thirty minutes.)

The objects having been covered, the excess of liquid is removed by means of blotting paper, and the following stain is allowed to flow in:

Glycerin	1 part.
Water	1 ,,
Picro-carmine	1 ,,

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[1] 'Journ. Roy. Mic. Soc.,' i (1878), p. 189. ('Journ. de Micrographie.')

[2] 'Comptes rendus,' 1879, p. 433 (1r sem.).

(Eosin may also be used. Soluble anilin-blue does not give such good results.) The stain should be placed at the edge of the cover and the slide put away in a moist chamber in order that the water may evaporate very slowly and be changed very gradually for the glycerin-mixture; if this precaution is not taken, shrinkage may occur. When the exchange has taken place, strong glycerin may be added and gradually substituted for the dilute glycerin.

Certes states that the organisms thus prepared are fixed perfectly in their natural form, and allow of the study of the minutest detail of cilia, flagella, and the like, with the highest powers; the green colouration of Euglenæ and Paramecia is preserved. The nuclear structures are sharply brought out by the picro-carmine.

639. Protozoa (Nuclei) (*Maupas' method*[1]).—Fix on a slide by means of a drop of alcohol drawn under the cover. Then draw in water, then saturated solution of picro-carmine, and then, after a few minutes, glacial acetic acid. Glycerin being then added, a permanent mount is obtained.

Instead of alcohol, exposure for one minute to the vapour of a 1 per cent. solution of osmic acid may be used for fixing; but the action of the picro-carmine must then be prolonged.

(*Stentor*, *Kondylostoma*, *Spirostomum*, &c., also for protophyta, *Volvocina*, *Fungi*, &c.) The special object of the method is the demonstration of nuclei.

640. Infusoria-staining—Cyanin or Quinoléin-Blue (*Certes' method*[2]).—Cyanin or quinoléin-blue is imperfectly soluble in water. A weak solution, however, suffices to stain Infusoria pale blue, without any immediate toxic action. In strong solutions it immediately destroys them. In the weak solutions employed by Certes (1·100,000), Infusoria lived for twenty-four or thirty-six hours.

For the staining of lymph-corpuscles, the cyanin may be dissolved in serum (which is a better solvent of it than water).

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[1] Ibid., p. 1276, (1r sem.), and p. 251 (2me sem.).

[2] Ibid., 1881 (1r sem.), p. 425.

The stain appears to be concentrated in the fatty granules of the protoplasm. Nuclei and nucleoli remain unstained. For this reason, the figures of nuclear division are very clearly brought out in contrast with the surrounding stained protoplasm. Certes considers quinoléin an important help to the study of cell-division, conjugation, and other minute phenomena of cell-life, in *living cells*.

The reactions on tissues or Infusoria *that have been killed* are of a quite different nature, and do not at present appear to be of any value.

In a later communication ('Zool. Anz.,' No. 84, p. 288 (1881), Certes announces that solutions of 1·500,000 have sufficient colouring power. He notes that the aqueous solutions should always be prepared with spring-water, as distilled water is a poison for Infusoria.

Alcoholic solutions (in $\frac{1}{3}$ alcohol) should be of 1·100,000 strength.

All solutions should be kept in the dark, as light decolourises them.

641. Methylen-Blue for Protozoa (*Certes' method*[1]).—A drop of alcoholic solution is placed on a slide and allowed to evaporate. When the evaporation is nearly complete, add a drop of the liquid containing the organisms. As soon as the staining action is complete, which quickly happens, the drop must be caused to flow away from the spot where the crystals are deposited, and may then be covered and examined.

Methylen-blue is an agent that stains living protoplasm.

642. Infusoria (*Géza Entz's methods*[2]).—For fixing, any of the usual fixing solutions may be employed, but picro-sulphuric acid is most to be recommended. Entz adds a few drops of the liquid to a watch-glass containing the animals in water. After one or two minutes the liquid is poured off, and the preparation washed out for about half an hour with not too strong alcohol. The objects may then be mounted in a mixture of equal parts of glycerin and water. But it is better to stain them before mounting. To this end they may be brought from the alcohol for ten to twenty minutes into picro-carmine (forms provided with a testa, such as *Euglena spirogyra*, *Phacus*, *Peridina*, require several hours' staining). Wash with water until the picrin is extracted.

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[1] Ibid., 1882 (2me sem.), p. 464.

[2] 'Zool. Anz.,' No. 96 (1881), p. 575.

643. Infusoria (*Korschelt's methods*[1]).—The organisms are fixed on the slide by means of a drop of 1 per cent. osmic acid run under the cover and drawn off at the opposite side. This is followed by successive drops of water, 70 per cent. alcohol, 90 per cent. alcohol, and water. The preparation is then stained for one and a half to two hours, in a moist chamber, with a drop of Weigert's picro-carmine. Wash out with 70 per cent. alcohol, followed by 90 per cent. and absolute alcohol, clear with clove oil, and mount with balsam.

For *Amœbæ* the osmic-acid treatment is not successful; for such organisms, 2 per cent. chromic acid may be used instead. It should be allowed to act for two to three minutes.

Landsberg[2] objects to the cover-glass as hindering the manipulations, and prefers to treat the organisms separately, taking them up by means of a capillary tube, and ejecting them from it into a drop of the reagent placed on a slide. (A small drop of liquid should be drawn up into the capillary tube before placing it near the object to be taken up, in order to diminish the violence of the rush-up of liquid.)

For *Actinosphærium*, Landsberg recommends glycerin instead of balsam.

644. Infusoria (*Blanc's methods*[3]).—For fixing, picro-sulphuric acid of the following composition:

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[1] Ibid., No. 109 (1882), p. 217.

[2] Ibid., No. 114, p. 336.

[3] Ibid., No. 129 (1883), p. 22.

Saturated picric-acid solution . .	100 vols.
„ sulphuric acid . . .	2 „
Water	600 „

This liquid is for larvæ of Echinodermata, of Medusæ, and of Porifera; for Rhizopoda and Infusoria, add two or three drops of 1 per cent. acetic acid for every 15 c.c. of the liquid. The acetic acid is added in order to bring out the nuclei and "nucleoli." Blanc fixes under a cover-glass, notwithstanding the objections of Korschelt. Wash out with 80 per cent. alcohol, followed by 90 per cent. and absolute. Stain with saffron solution (Formula No. 106), wash out with 80 per cent. alcohol until the colour is sufficiently extracted, and pass through absolute alcohol into clove oil.

Blanc recommends the method for the preservation of most microscopic organisms, and in particular for marine *Nematoda*, the stain being sufficiently penetrating to pass through their thick chitinous integument.

645. Gaule's "Würmchen" (or "Cytozoa") (*Drepanidium ranarum*, LANKESTER) (*Gaule's method*[1]).—Choose a medium-sized *vigorous* frog, with clear eyes and lively movements. (Gaule's experiments were chiefly made with *R. esculenta.*) The frog must not have been long kept in confinement, and he must have been kept in a warm place for some hours before the experiment. Prepare a stoppered glass cylinder containing a few c.c. of quicksilver and 5 c.c. of 0·6 per cent. salt-solution. With scissors make a cut from the tympanum of the frog on either side towards the backbone (do not incise the backbone), and allow the blood to flow into the salt-solution. As soon as the blood gets to drop slowly the frog may be killed and thrown away. Then close the cylinder and agitate for a few moments, until the quicksilver appears broken into very fine drops on the sides of the vessel. Allow the quicksilver to settle, remove a drop of the supernatant fluid with a pipette, mount it on a slide, and close the cover with paraffin. Put the preparation on a hot stage already warmed. As to temperature, Gaule cannot give *absolute* directions, as the temperature of *the preparations placed on* any form of hot stage is of course unknown; he can only state that, using a Max Schultze's stage, the proper temperature was found to be attained when the thermometer stood between 30° and 32° C.; he advises in no case to go beyond 32°. (The function of the quicksilver is, of course, to defibrinate the blood by agitation. The concentration of the NaCl solution may be varied, though not beyond the limits of from 0·3 to 3 per cent. It is important to remember that the appearance of the Cytozoa is a periodical phenomenon.[1] In *November* all frogs of over 50 grammes weight had Cytozoa, whilst all frogs of less than 50 grammes were without them, whilst in *May* frogs of under 50 grammes possessed them, and those of over 50 grammes were without them. The *maximum* of Cytozoa in large frogs is found in autumn, in small frogs in spring. Gaule never found any in frogs under 20 grammes.)

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[1] 'Arch. Anat. u. Phys.,' 1880 (Phys. Abth.), p. 60.

The Cytozoa exist in quantities in liver, marrow of bone, and spleen. They may be studied with the greatest ease by simply teasing a piece of spleen with 0·6 per cent. salt-solution, when they may at once be recognised in the spleen-cells.[2]

Instead of 0·6 per cent. salt-solution it is sometimes useful to employ a solution containing 0·3 per cent. chloride of potassium, and 0·3 per cent. chloride of sodium.[3]

Good results are also obtained by adding to the 0·6 per cent. salt-solution a trace of gentian-violet. After twelve to twenty-four hours the blood-corpuscles will be found decoloured and stained, and may be treated with osmic acid and dried for preservation.

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[1] Ibid., 1881 (Phys. Abth.), p. 303.

[2] 2nd method, ibid., p. 307.

[3] 3rd method., ibid., p. 312.

CHAPTER XLVIII.

VARIA.

646. Immobilisation of Batrachians (*Robin's methods*[1]).—Subcutaneous injection of a few drops of chloroform or of solution of curare; or a small piece of curare in substance may be placed under the integuments. Frogs may also be anæsthetised by placing them in water containing a few drops of chloroform or ether.

Mammalia.[2]—Inject curare under the skin and study the circulation of the mesentery.

Annelida.[3]—Similar processes may be employed with Náis, &c.

647. Curare (*Schmuziger's formula*[4]).—To immobilise frogs for the study of the circulation, ½ to ¾ centigramme of a 0·1 per cent. curare solution is injected. Complete immobility is obtained after the lapse of 1½ to 1¾ hours. The observations may sometimes be continued for more than fifty hours.

648. Alcohol as an Anæsthetic (*Rudneff's method*[5]).—Introduce 2 or 3 drops of spirit of wine into a frog's mouth.

649. Ether as an Anæsthetic (*Gage's method*[6]).—In order to quiet live aquatic animals, add a small quantity of sulphuric ether to the water in which they are kept. Ether mixes readily with water, does not sensibly affect the circulation, and the animals are as lively as ever soon after being put back into fresh water.

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[1] Robin, 'Traité,' p. 6.

[2] Ibid.

[3] Ibid.

[4] 'Arch. Mik. Anat.,' ix (1873), p. 709.

[5] Ibid., i (1865), p. 295.

[6] 'American Quart. Micro. Journ.,' i, p. 71. 'Journ. Roy. Mic. Soc.,' i (1878), p. 353.

650. Cleaning Slides and Covers (*Hanaman's formula*[1]).—To a cold saturated solution of bichromate of potash, add $\frac{1}{8}$ of its bulk of strong sulphuric acid (care must be taken on account of the heat and vapours evolved).

651. Cleaning Cover-glasses (*Heneage Gibbes's method*[2]).—Place the cover-glasses in strong sulphuric acid for an hour or two, wash well until the drainings give no acid reaction; wash first with methylated spirit, and then with absolute alcohol, and wipe carefully with an old silk handkerchief.

652. Cleaning Slides and Covers (*Seiler's method*[3]).—*New* slides and covers are placed for a few hours in the following solution:

Bichromate of potash .	2 ounces.
Sulphuric acid . .	3 fluid ounces.
Water	25 ,,

Wash with water. The slides may be simply drained dry; the covers may be wiped dry with a linen rag.

Slides and covers that have been used for mounting either with balsam or a watery medium are treated as follows:—The covers are pushed into a mixture of equal parts of alcohol and hydrochloric acid, and after a few days are put into the bichromate solution and treated like new ones. The slides are scraped free of the mounting medium with a knife and put directly into the bichromate solution.

653. Sulpho-Cyanides of Ammonium and Potassium (*Stirling's methods*[4]).—The action of a 10 per cent. solution of these salts was investigated in the case of many tissues. In almost all cases it was found to be useful for demonstrating the intra-nuclear fibrillar plexuses of cells. It would appear that some interfibrillar material is acted upon and swells up so as to separate the fibres that form the meshwork, and that the refractive index of the latter is so altered that their arrangement can easily be made out. The plexus thus revealed may be stained with fuchsin, eosin, or picro-carmine, but frequently it will not be found necessary to stain.

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[1] 'Journ. Roy. Mic. Soc., i (1878), p. 295. 'American Naturalist,' xii, p. 573.

[2] Ibid., iii (1880), p. 392.

[3] Ibid., p. 508.

[4] 'Journ. of Anat. and Phys.,' xvii (1883), p. 208.

The solution is an admirable dissociating medium for epithelium. (Macerate small pieces for twenty-four or forty-eight hours; stain as above.)

The action of these salts on the lens is quite peculiar. After being acted on for twenty-four to forty-eight hours (macerated in 10 per cent. solution) the lens-fibres become beaded or moniliform.[1]

Retina.—After maceration for twenty-four hours in a sulphocyanide solution, the outer segments of the rods (frog) reveal transverse striation very distinctly, and the same happens with the large cones of fishes.

654. Chain or "Ribbon" Section-cutting.—It is probably a familiar fact to the majority of workers with the Thoma microtome that if a series of paraffin sections be cut in succession and not removed from the knife one by one as cut, but allowed to lie undisturbed on the blade, it not unfrequently happens that they adhere to one another by the edges so as to form a chain which may be taken up and transferred to a slide without breaking up, thus greatly lightening the labour of mounting a series. The following appear to me to be the factors necessary for the production of a chain. Firstly, the paraffin must be of a melting-point having a certain relation to the temperature of the laboratory. I have not had time to carry out a series of experiments intended to settle the melting-point of the paraffins that should be used at the different temperatures at which sections are usually cut, but I can indicate at least one point with considerable accuracy. *Small* sections can always be made to chain when cut from a good paraffin of 45° C. melting-point in a room in which the thermometer stands at 16° to 17° C. (The temperatures quoted apply to the case of rooms heated by an open fire, and probably would not apply to the case of rooms heated by closed stoves, such as are usual in Germany.) At 15° C. the paraffin will be found a trifle hard. At 22° C., the proper melting-point of the paraffin will probably be found at about 48° C., but my observations at these temperatures are less extended. Secondly, the knife should be set square. Thirdly, the block of paraffin should be pared-down very close to the object, and should be cut so as to present a straight edge parallel to the knife edge; and the opposite edge should also be parallel to this. The block should in no case be cut so as to present a pointed side, as recommended by the Naples Zoological Station. Fourthly, the sections ought to be cut rapidly, with the swiftest strokes that can be produced. It is evident that this condition can only be conveniently realised by means of a sliding microtome; but it is by no means necessary to have recourse to special mechanical contrivances, as in Caldwell's automatic microtome. The Thoma microtome well flooded with oil is sufficient.

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[1] Ibid., p. 209.

655. Parabolic Mirror for Correction of too Hard or too Soft Paraffin (*H. Fol's method*[1]).—If, after cutting has begun, the paraffin be found to be too hard, it may be softened by the following simple and ingenious expedient:—A lamp provided with a parabolic reflector is set up near the microtome in such a position that the heat-rays of the flame are thrown by the reflector on to the imbedded object. The right temperature is obtained by adjusting the distance of the lamp.

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[1] Fol, 'Lehrbuch d. vergl. mikr. Anat.,' p. 123. Leipzig, Engelmann, 1884.

If, on the contrary, the paraffin be found too soft, it may be hardened by exposing it to the cooling influence of a lump of ice placed in the focus of a similar reflector.

It is often sufficient to moderate the temperature of the room by opening or closing the window, stirring the fire, setting up a screen, or the like.

656. Ribesin (*H. Fol's formula*[1]).—The juice of black currants (*Ribes nigrum*) having been expressed and thrown away, the skins are boiled for some hours in 10 per cent. alum-solution. The resulting deep violet solution may conveniently be diluted with water, and after the lapse of a day should be filtered, and may then be used for staining.

The stain resembles in its effect that of Boehmer's hæmatoxylin, but is a still more precise nuclear stain. It is a bright, somewhat greenish blue, agreeable, distinct, and permanent. Alcohol objects stain quicker than chromic acid ones, but the most suitable of all are bichromate objects.

657. Ribesin and Eosin (*H. Fol's formula*[2]).—A ribesin stain may be followed by eosin-staining, or a double stain may be at once obtained by adding a little eosin to the above-described ribesin-solution and filtering (the filtrate should be cherry red). Wash out with alcohol charged with a little eosin, and clear with clove oil also charged with eosin. The blue of the ribesin remains fixed in the nuclei. In many respects a better double stain than Renaut's hæmatoxylic eosin.

658. Perchloride of Iron as a Fixing-agent (*H. Fol's formula*[3]) (Addendum to No. 35).—After further experiment with this most important fixing-agent, Fol now recommends the following proportions:—One volume of tincture of perchloride of iron (B.P.) diluted with 5 to 10 volumes of 70 per cent. alcohol. (If in course of time a fine precipitate makes its appearance, add a drop or two of HCl and agitate.) After fixing, wash out with 50 per cent. alcohol containing ½ to 1 per cent. of oxalic acid until the yellow colouration is entirely removed. The objects may then be washed with pure alcohol and stained with carmine or any of the usual stains.

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[1] Ibid, p. 183.
[2] Ibid, p. 196.
[3] Ibid, p. 102.

If it be preferred to avoid treatment with alcohol, an aqueous solution of oxalate of potash may be employed in the place of the alcoholic solution of oxalic acid.

This reagent fixes with great rapidity and precision, but is not so penetrating as might be imagined, for which reason the portions of organs to be fixed should be taken as small as possible.

659. Erlicki's Solution (Addendum to No. 213).—This modification of Müller's solution is known in Germany as Erlicki's solution.

660. Carbolized Syrup. Carbolized Glycerin (*Déclat's formulæ*) (Addendum to Nos. 294 and 342).—These most elegant pharmaceutical preparations have the great advantage of being perfectly colourless. They may be obtained at the Déclat Company's Depôts, 49, Southwark Street, London, 183, Broadway, New York, and 6, Avenue Victoria, Paris. The carbolized glycerin ("Glycophénique") contains 10 per cent. carbolic acid; the syrup is a much weaker solution, but still sufficiently concentrated to ensure perfect preservation.

ERRATUM.

P. 192, line 5 from bottom, *for* 7 grammes *read* 7 parts.

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PRINTED BY J. E. ADLARD, BARTHOLOMEW CLOSE.

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